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Prolongation of Pseudopregnancy by Induction of Deciduomata in the Rat.

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Pseudopregnancy is a condition characterized by many of the phenomena of early pregnancy: a prolonged leucocytic vaginal smear, presence of large functional corpora lutea, reduced spontaneous activity, increased body weight, increased food consumption, development of the mammary glands, and progestational changes in the uterine mucosa with susceptibility to deciduomata formation. In the rat its length is subject to considerable variation but its duration is usually 13 to 15 days. Long and Evans[1] from an analysis of 151 cases have pointed out that the average duration of this condition from its induction to the succeeding estrus was 14.38 days. Slonaker[2] in an analysis of 475 cases found the average duration to be 14.53 days. This represents a material lengthening of the estrous cycle but is still considerably short of the period of pregnancy.

Maintenance of luteal function is essential throughout pregnancy in the rat. This is borne out by the fact that bilateral ovariectomy invariably terminates gestation.[3-6] Removal of the pituitary gland during the first half of pregnancy is likewise followed by death and resorption of the fetuses, whereas pregnancy continues if hypophysectomy is performed after the tenth or eleventh day.[7,8] This indicates that maintenance of corpora lutea is under the control of the pituitary gland (more specifically of lactogenic hor-

* With the Research Laboratories of Emory W. Thurston.

1 Long, J. A., and Evans, H. M., *Memoirs of the Univ. of Calif.*, 1922, **6**.

2 Slonaker, J. R., *Am. J. Physiol.*, 1929, **89**, 406.

3 Harris, R. G., and Pfiffner, J. J., *Anat. Rec.*, 1929, **44**, 205.

4 Johnson, G. E., and Challans, J. S., *Anat. Rec.*, 1930, **47**, 300.

5 Nelson, W. O., and Haterius, H. O., *Physiol. Zool.*, 1930, **3**, 231.

6 Hain, A. M., *Quart. J. Exp. Physiol.*, 1934, **24**, 101.

7 Pencharz, R. I., and Long, J. A., *Am. J. Anat.*, 1933, **53**, 117.

8 Selye, H., Collip, J. B., and Thompson, D. L., PROC. SOC. EXP. BIOL. AND MED., 1933, **30**, 589.

mone[9]) for the first half of pregnancy; but during the second half is controlled by some extra-pituitary factor, elaborated according to Astwood and Greep[10] by the fetal placenta. Inasmuch as the pseudopregnant rat lacks an extra-pituitary luteotrophin, pseudopregnancy terminates rapidly after cessation of lactogenic hormone secretion.

In the present experiment pseudopregnancy was prolonged to the period of normal pregnancy in the rat following traumatization of the endometrium and subsequent formation of deciduomata and metrial glands.

Material and Technic. Fifty-six female rats 3 to 4 months of age were used in the following experiment. Vaginal smears were taken daily for a period of 2 weeks and the regularity of the estrous cycle determined. Animals showing fully cornified vaginal smears were selected on the first day of an experiment. The cervix uteri of each rat was stimulated for 1 or 2 seconds by an alternating current of 60 volts. Four days after the cervical stimulus was applied, the endometrium of the right uterine horn was traumatized by inserting a silk thread for a distance of 1 or 2 cm through the uterine lumen. Animals were autopsied (8 in a group) 4, 6, 8, 10, 13, 16, and 18 days after endometrial traumatization. Uteri and ovaries were fixed in formol, and sections prepared stained with hematoxylin and eosin and Mallory fluid. In addition 24 animals of similar age and weight were made pseudopregnant but their endometrium was not traumatized. Vaginal smears were taken daily in all cases, and the length of time from induction of pseudopregnancy until the succeeding estrus determined. Whole mounts of mammary glands were also prepared.

Results. In animals whose endometrium was not traumatized the period of pseudopregnancy (from electrical stimulation to the subsequent vaginal cornification) was 13.7 days (range, 12-15 days). In animals whose endometrium was traumatized, vaginal cornification did not recur before the twenty-second day (18th day post-traumatization).† Associated with this prolongation of pseudopregnancy were maintenance of the corpora lutea,‡ inhibition of follicular development (as determined histologically), leucocytic vaginal smears, and a structure in the insertion region of the uterus which Selye and McKeown[11] have termed "the metrial gland." The first evidence of metrial gland formation was observed 6 days post-traumatization.§ The gland attained maximum diameter 10 days post-traumatization and regressed subsequent to this time. By the sixteenth day (20 days after the induction of pseudopregnancy) the structure was still grossly visible and well developed.‖ Even by the eighteenth day gross enlargement of the insertion region marked the site of the metrial gland although typical "metrial cells" were not observed.

Mammary glands developed in the traumatized pseudopregnant rat to the same extent as they did in the untraumatized animal. The presence of deciduomata and metrial glands did not prevent mammary regression subsequent to the twelfth day of pseudopregnancy.

Summary. The induction of deciduomata resulted in prolongation of pseudopregnancy to the period of normal pregnancy in the rat. This prolongation occurred in the absence of fetal tissue and was associated with the presence of metrial glands, maintenance of corpora lutea, and inhibition of follicular development.

9 Lyons, W. R., Simpson, M. E., and Evans, H. M., PROC. SOC. EXP. BIOL. AND MED., 1943, **52**, 134.

10 Astwood, E. B., and Greep, R. O., PROC. SOC. EXP. BIOL. AND MED., 1938, **38**, 713.

† On this day 5 of the 8 animals in this group came into estrus.

‡ Attempts to induce secondary deciduomata were unsuccessful. Traumatization of the untraumatized horn 4, 6, 8, or 10 days subsequent to initial tranmatization failed to induce a decidual growth although metrial glands were present at the site of original traumatization.

11 Selye, H., and McKeown, T., *Proc. Royal Soc. B.*, 1935, **119**, 1.

§ Development of the metrial gland occurred at the same time as decidual regression. First evidence of decidual regression was observed 6 days post-traumatization. On the 8th and 10th day post-traumatization decidual necrosis was increasingly more evident. By the 13th day post-traumatization no decidual stroma cells were observed.

‖ Administration of trypan blue revealed that this dye was not ingested by the metrial cell before the 16th day post-traumatization.

14351

Negative Conditioned Reflexes Versus Absence of Response Elicited from External Inhibition.*

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In connection with a study of the association areas of the cerebral hemispheres which are essential for obtaining correct conditioned differential responses of a foreleg with olfactory, auditory, general cutaneous and optic stimuli, a correct absence of response appeared in some of the early negative tests with normal and operated dogs, which at first was confused with a later well-differentiated negative conditioned reflex.

Clarification of this reflex requires familiarity with the technic used in the general problem, as follows: Flexion of the foreleg is the conditioned response, to be correctly positive it must occur within 7 seconds, to be correctly negative it must be withheld for 7 to 15 seconds, depending on the time of appearance of the positive reflex. To be called correct, conditioned differential responses required at least 95% correctness when the positive and negative stimuli were given in varied order. A positive or negative conditioned reflex was never reinforced except for error, in which case the positive error was punished by electric shock and the negative by whipping. Emphasis therefore was placed on the correctness of the reflexes rather than on fine discriminations between analysers.

Two sets of correct conditioned differential tests were used for each sense, one based on components and the other on rate. For example in one set of auditory tests the positive conditioned stimulus consisted of tapping a bell once per second and the negative consisted of tapping a board once per second. In the other set, a bell was tapped once per second for the positive stimulus and 3 times per second for the negative stimulus.

A correct negative reflex that could be elicited by *external inhibition*[1] is illustrated by the following example, which is but one from many normal dogs in which the first negative conditioned test resulted in absence of response to an auditory, olfactory, or general cutaneous stimulus. After having acquired the positive conditioned reflex perfectly for the bell tapped once per second, this dog failed to flex his foreleg during the first negative conditioned test for tapping a board. A simultaneous thoracic record (Fig. 1, C)[2] disclosed inhibition of respiration, and there was complete inhibition of all movements. The second negative conditioned test also resulted in absence of foreleg responses. These tests, however, were followed by many other negative tests mixed in varied ratios with positive conditioned tests, and all tests resulted in positive responses, with respiration showing as much excitation for the incorrect negative responses as for the correct positive reflexes, in spite of the punishment which followed the errors. Finally the true negative reflexes appeared and both negative and positive reflexes were perfect under all conditions.

The difference between the genuine negative conditioned reflex and the early negative reflex due to external inhibition resulting from the marked contrast of the stimuli was frequently well illustrated during the correct conditioned differential tests with olfactory sense after prefrontal lobectomy, and during the general cutaneous and auditory differential tests after removal of large portions of the association cortex. A typical example from a dog previously trained to make correct conditioned differential responses with 2 sets of auditory analysers, namely, bell and iron cup, and bell tapped once per second and 3 times per second, was tested after prefrontal lobectomy and removal of a large portion of the posterior association cortex from one hemisphere (All projection centers but optic were intact). The conditioned differential reflexes

* Aided by a grant from the John and Mary R. Markle Foundation.

[1] Pavlov, I. P., *Conditioned Reflexes*, Trans. C. V. Anrep, 1927.

[2] Allen, W. F., *Am. J. Physiol.*, 1942, **137**, 783.

based on different rates of the bell were tested first, with the result that it required 200 positive tests intermixed with 96 negative tests before any sign of regularity occurred in the correct responses to the negative conditioned stimuli. In the first 8 sessions of testing after the operations practically all of the positive tests were correct and nearly all of the negative tests were positive and incorrect. The greatest number of correct negative tests came during the first test session, when 3 out of 12 were correct, the first, second, and fourth. Had the results of the early negative tests been listed as true negative conditioned reflexes, and only a few tests been made, this dog might have been reported as retaining its ability to make correct differential responses in spite of its extirpated cortex.

Another typical example of an external inhibition reflex may be cited from a dog which had acquired correct conditioned differential responses with all the routine analysers before the operation, but which after prefrontal lobectomy and bilateral removal of large portions of the association cortex (all projection centers except optic were intact) was unable to acquire correct conditioned differential responses with either set of general cutaneous analysers. In some 700 tests after operation practically all of the positive tests were correct and a large proportion of the negative conditioned tests were positive responses and incorrect. There were, however, 1 or 2 correct negative tests during each day's session, and these could be explained as the result of external inhibition since in these tests there was inhibition of respiration and other movements. The entire picture of every daily session of tests after the operations was one of inability of the dog to make correct differential responses.

That these two negative reflexes are really different is indicated by the following observations: (1) The negative reflex from external inhibition occurs in normal dogs long before there are any signs of correct conditioned differential responses. (2) They may appear in dogs after varying amounts of association cortex have been removed, when correct conditioned differential negative reflexes cannot be executed. (3) The external inhibition reflex is more than mere cessation of nervous activity, for respiration is inhibited and a restless animal becomes quiet. This reflex is apparently simpler than the negative conditioned response and may involve fewer neurons.

These early sporadic inhibitory reflexes have not been observed in all our dogs during the process of acquiring correct conditioned differential responses, but when they do occur it is important that they are recognized.

14352 P

Development of Cirrhosis in the Liver of Dogs Deprived of Both Pituitary and Thyroid Glands.*

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It was shown recently that the concentration of all lipid constituents (total fatty acids, phospholipids, free and esterified cholesterol) is greatly increased in the blood of the hypophysectomized-thyroidectomized dog.[1] The present report deals with the condition of the livers of such animals.

The livers of 9 dogs were examined at intervals of 70 to 419 days after they had been deprived of both pituitary and thyroid glands. The completeness of hypophysectomy in all dogs was established at necropsy. A fatty infiltration of the liver was observed in 8 of them. The livers of 6 contained abnormal

* Aided by a grant from the Christine Breon Fund for Medical Research.

[1] Entenman, C., Chaikoff, I. L., and Reichert, F. L., *Endocrinology*, 1942, **30**, 802.

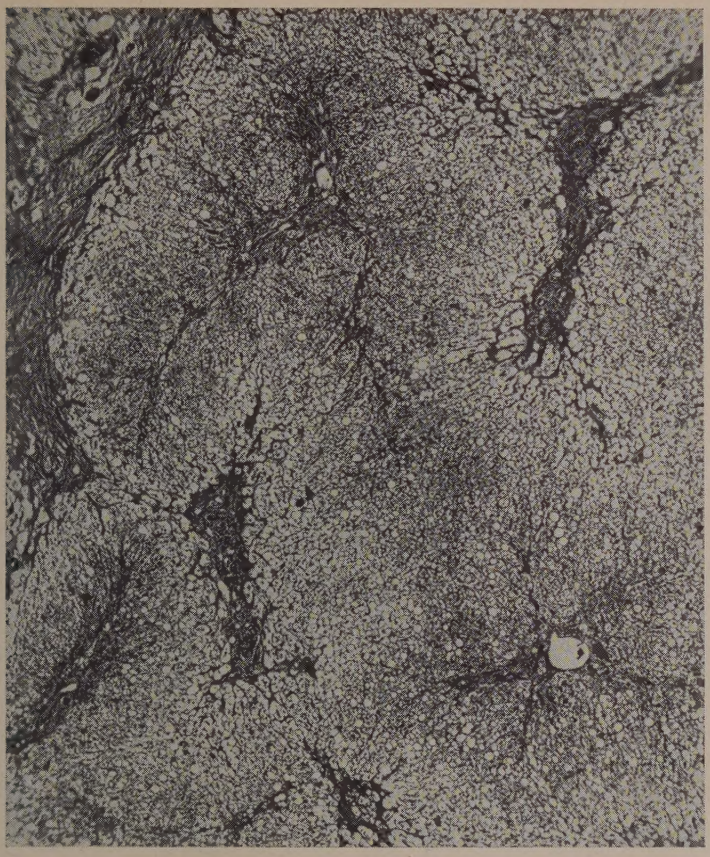

FIG. 1.
Microphotograph of section of liver of dog HT8. Stain, hematoxylin and eosin. Magnification, 73.

amounts of fibrous tissue; all degrees of cirrhosis from early to advanced portal cirrhosis were found. The diet received by these dogs was adequate with respect to calories, proteins, salts, and vitamins and not high in fat. The details of the diet have been recorded elsewhere.[1]

A section from a typical liver (dog HT8) is shown in Fig. 1. Excessive amounts of fat are present in the liver cells. The fat is distributed in both large and small droplets. There is a well developed cirrhosis with excessive amounts of fibrous connective tissue in the portal spaces. Fine ramifications of connective tissue radiate outward from the portal areas, in some instances connecting one area with another.

The high fat content (11 to 52%; average 21%) found in the livers of the hypophysectomized-thyroidectomized dogs is in keeping with previous reports from these laboratories in which it was shown that a fatty infiltration precedes the formation of fibrous tissue in the liver of the dog.[2,3] Cirrhosis of the liver was not observed in dogs subjected to thyroidectomy alone but in these animals the fat content of the liver did not exceed 11%.

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2 Chaikoff, I. L., Connor, C. L., and Biskind, G. R., *Am. J. Path.*, 1938, **14**, 101.

3 Chaikoff, I. L., Eichorn, K. B., Connor, C. L., and Entenman, C., *Am. J. Path.*, 1943, **19**, 9.

14353

Relationship of the Virus of Cat Pneumonia (Baker) to the Psittacosis-Lymphogranuloma Group of Agents.*

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An infectious respiratory disease of domestic cats has been shown by Baker[1] to be associated with the presence of a virus which is characterized by the formation of elementary bodies. This agent was recovered from suspensions of infected cat lung by the intranasal inoculation of mice. Following inoculation of previously uninfected kittens it produced an acute respiratory disease with pneumonia. The virus was highly pathogenic for mice and could be passed readily in this species by intranasal inoculation. It was easily cultivated in the yolk sac of developing chick embryos. Impression films of infected lung tissue or of yolk sac membranes revealed numerous elementary bodies when either the Giemsa or the Macchiavello stain was used. Immunological studies with this virus have not as yet been reported.

Early in the course of work on the Baker virus it became apparent that this agent possesses certain characteristics similar to those of the virus of mouse pneumonia reported by Nigg.[2] The latter also is an elementary-body virus which is known to be antigenically related to the psittacosis-lymphogranuloma group.[3] The Baker agent, like the Nigg virus, was found to be exclusively pneumotropic in its pathogenicity for mice, producing no evidence of disease when inoculated intracerebrally or intraperitoneally. The intranasal inoculation of low dilutions of either virus into mice usually resulted in extensive pneumonia and death within 48 hours. Higher dilutions, on the other hand, caused the development of small areas of pale grey consolidation which slowly increased in extent and sometimes persisted for as long as 2 months after inoculation. The pathological lesions in the lungs of mice inoculated with these 2 agents were indistinguishable microscopically. Because of these general similarities, a study was made of the possible antigenic relationship of the Baker virus to the Nigg virus and to the virus of psittacosis. In this paper, evidence is presented which indicates that the Baker virus, like the Nigg virus, should be regarded as a member of the psittacosis-lymphogranuloma group. A comprehensive comparison of the biological properties of the Baker and Nigg viruses will be the subject of a subsequent report.

Viruses. A strain of the cat pneumonia virus in yolk sac material was obtained through the courtesy of Dr. James A. Baker. The virus was maintained by passage in the yolk sacs of developing chick embryos and by passage in the lungs of mice. In order to avoid the possibility of contamination of the agent with a latent mouse virus, egg passage material was used exclusively for the immunization of animals and for the preparation of antigens for complement-fixation tests.

Through the courtesy of Dr. Geoffrey Rake, the Nigg mouse pneumonia virus was obtained in the form of infected mouse lung tissue. Three serial passages in Swiss mice were carried out in this laboratory, following which the virus was maintained by passage in eggs. A strain of psittacosis virus, which was isolated from an infected pigeon in this laboratory during 1942,[4] was used.

Complement-Fixing Antigens. Baker and Nigg virus antigens were prepared from infected yolk sacs by a method similar to that

* The Bureau of Medicine and Surgery of the United States Navy does not necessarily undertake to endorse views or opinions which are expressed in this paper.

1 Baker, James A., *Science*, 1942, **96**, 475.

2 Nigg, C., *Science*, 1942, **95**, 49.

3 Eaton, M. D., and Corey, M., PROC. SOC. EXP. BIOL. AND MED., 1942, **51**, 165.

4 Smadel, J. E., Wall, M. J., and Gregg, A., *J. Exp. Med.*, 1943, **78**, 189.

described by McKee, Rake and Shaffer[5] for *Lymphogranuloma venereum* virus. Psittacosis virus antigen was prepared from chick embryo tissue culture infected with a parakeet-derived strain of psittacosis virus. The method used for the preparation of this antigen has been described by Smadel.[6] Control antigens were prepared from normal yolk sacs and from uninfected tissue culture material by methods identical with those used for the virus antigens.

Sera. Immune sera against the Baker and Nigg viruses were obtained from Syrian hamsters or from mice which had been inoculated intranasally with infected egg material. Antiserum against the pigeon strain of psittacosis virus was obtained from mice which had received intraperitoneal injections of a suspension of infected mouse brain. Convalescent serum from 2 patients who had recovered from psittacosis were included in complement fixation tests against the various antigens.

Complement-fixation Technic. The complement-fixation technic employed was identical in all details with that previously described.[7] Sera were tested in two-fold dilutions, from 1-4 to 1-256. The endpoint in each test was taken as the highest dilution of serum in which little or no hemolysis occurred. The titers were recorded in terms of the initial dilution of serum. Appropriate hemolytic and anticomplementary controls were included in each test. All reagents were tested for anticomplementary activity in twice the highest concentration used in a test. Comparisons of the activity of antisera and antigens were made simultaneously in the same experiment. Normal human and animal sera, as well as uninfected yolk sac and tissue-culture antigens, were included as controls in each test.

Results. Complement-fixation tests were carried out with Baker, Nigg, and psittacosis viruses in the presence of antisera against each of these agents. The results of these tests are presented in Table I. It will be seen that the antisera against the Baker virus fixed complement in high dilution in the presence of both the psittacosis and Nigg virus antigens. The titers observed against these heterologous antigens were not significantly lower than the titer against the Baker virus antigen itself. Similarly, the antisera against psittacosis and Nigg viruses produced fixation of complement with the Baker virus antigen. It is noteworthy that both convalescent sera from patients with psittacosis also fixed complement at similar dilutions with all 3 virus antigens. No significant antigenic differences between the 3 viruses were revealed by these tests when either hamster or human serum was employed. However, in the case of immune mouse serum it is of interest to note that the titer against the homologous virus was usually considerably higher than was the titer against either of the heterologous viruses.

The results of these tests indicate that the virus of cat pneumonia (Baker) is antigenically related to psittacosis virus and to

[5] McKee, C. M., Rake, G., and Shaffer, M. F., PROC. SOC. EXP. BIOL. AND MED., 1940, **44**, 410.

[6] Smadel, J. E., *J. Clin. Invest.*, 1943, **22**, 57.

[7] Thomas, L., Curnen, E. C., Mirick, G. S., Ziegler, J. E., Jr., and Horsfall, F. L., Jr., PROC. SOC. EXP. BIOL. AND MED., 1943, **52**, 121.

TABLE I.
Results of Complement-Fixation Tests with Baker, Nigg, and Psittacosis Viruses and Antiserum Against Each of These Agents.

Antiserum		Complement-fixation titer against indicated antigen				
Species	Immune against	Baker virus	Nigg virus	Psittacosis virus	Yolk sac control	Tissue culture control
Hamster	Baker virus	1-256	1-256	1-128	0	0
"	Nigg "	1-64	1-128	—	0	0
Mouse	" "	1-16	1-64	1-8	0	0
"	Psittacosis virus	1-32	1-16	1 64	0	0
Human (L)	" "	1-128	1-256	1-256	0	0
" (A)	" "	1-64	1-64	1-64	0	0
Hamster	Normal	0	0	0	0	0
Mouse	"	0	0	0	0	0
Human	"	0	0	0	0	0

the Nigg virus of mouse pneumonia. Minor antigenic differences between these 3 elementary body viruses may, however, exist.

Discussion. It seems apparent that another potential host, the cat, must be added to the wide variety of species from which viruses of the psittacosis-lymphogranuloma group have been recovered. Certain members of this group of elementary-body viruses have been tentatively separated from each other on the basis of minor antigenic differences,[3] differences in host range,[8] or differences in the route of inoculation by which they are pathogenic.[9] In mice the Baker virus is limited in its pathogenicity to the respiratory tract and in this respect it differs from the viruses of parrot or pigeon psittacosis, meningopneumonitis, and lymphogranuloma venereum, but resembles the Nigg virus of mouse pneumonia. The similarity between these two viruses is of considerable interest in view of the possibility that the disease in cats may have originated as the result of feeding upon infected mice. The recent observation by Karr[10] that normal mice could become infected following the ingestion of infected mouse tissue, may support this view.

Conclusion. The virus of cat pneumonia described by Baker is antigenically related to psittacosis virus and to the Nigg virus of mouse pneumonia. It should, therefore, be included in the psittacosis-lymphogranuloma group.

[8] Eaton, M. D., Beck, M. D., and Pearson, H. E., *J. Exp. Med.*, 1941, **73**, 641.

[9] Pinkerton, H., and Moragues, V., *J. Exp. Med.*, 1942, **75**, 575.

[10] Karr, H. V., *J. Inf. Dis.*, 1943, **72**, 108.

14354

Formation of Invisible, Non-perceptible Films on Hands by Cationic Soaps.*

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Many of the synthetic detergents of the cationic type are excellent germicides.[1] They also clean skin very effectively when employed in aqueous solutions at about one per cent concentration. While studying the effectiveness of the detergents in this concentration range for rapid degermation‡ of hands, we encountered several unexpected phenomena which are described in this report.

The extent of degermation was evaluated by a modification of the technic devised by Price.[2] Each hand is brushed systematically for 15 seconds over the nails, 15 seconds over the back of the hand, and similarly over the palmar surface. During this procedure the brush and hands are dipped frequently into 2 liters of water in a basin. Soap is added to the collecting basin to neutralize the cationic detergent and prevent bacteriostasis. One cc of this solution is poured with beef-infusion dextrose agar medium into a petri dish. The number of bacterial colonies on the plates is counted after incubation for 2 days, and the number of organisms removed from the hands is calculated. By this technic usually 1 to 10 million organisms are removed from both

* The work described in this paper was done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and the University of Chicago.

† On leave from University of Chicago for active duty in U. S. Public Health Service, Washington, D.C.

[1] Baker, Z., Harrison, R. W., and Miller, B. F., *J. Exp. Med.*, 1941, **74**, 611.

‡ "Degermation" includes both the killing and the mechanical removal of bacterial flora.

[2] Price, P. B., *J. Infect. Dis.*, 1938, **63**, 301.

hands during the 90 seconds scrub-up. With either soap or water, about the same number of organisms are brushed off the hands.

When the hands were scrubbed or dipped for one to 3 minutes in a one per cent solution of typical cationic detergent-germicides such as Zephiran or Phemerol,§ it was observed that the hands appeared to have been sterilized completely. However, when ordinary soap was then added to the hands many organisms were obtained (the soap employed was invariably sterile). The results of a typical experiment are shown in Table I.

TABLE I.
Apparent Sterilization of Skin by Zephiran.

Initial count	4,300,000
Hands then brushed for 3 minutes in one percent aqueous Zephiran	
Count obtained by 1½-minute brush-up *in water*	0
Count obtained by 1½-minute brush-up *with soap*	2,100,000

On the basis of these results and other evidence, described later, it has seemed to us that the cationic detergents deposit an invisible, non-perceptible film on the hands which retains bacteria under it. When the film is broken by chemical means, *e.g.*, by the addition of a negatively-charged detergent such as soap, then the organisms are released.‖

The durability of the film under favorable circumstances is quite unusual. For example, it has been found that subjects may use their hands for as long as 3 hours after the initial application of the detergent without disrupting the film. A typical experiment is shown in Table II.

TABLE II.
Durability of Film.

Initial count	4,270,000
Hands brushed for 3 minutes in 1:100 aqueous Zephiran. Subject then went about his business for 2½ hours, working with hands and fingers as usual.	
Count at 2½ hours (brush-up in *water* for 1½ minutes)	0
Count repeated immediately as above	0
" " " " "	10,000
" " " " "	80,000
Final count (brush-up in *soap* for 1½ minutes immediately after above)	2,200,000

Another unusual property of the film is the bactericidal power of the outer surface as contrasted to the inner surface. It was observed that many of the individuals who had been studied in experiments such as the one reported in Table II, did not contaminate their hands at their work during the several hours of the experimental period. Experiments designed to test the suspected bactericidal property of the outer surface have revealed a rapid, effective germicidal action when bacteria are placed on hands which have been covered previously with the detergent film. A typical experiment with *Staphylococcus albus* is shown in Table III.

TABLE III.
Bactericidal Properties of Outside of Film.

Count after scrubbing hands for 3 minutes with 70% alcohol (by weight)	20,000
Left hand dipped in 1:100 aqueous Zephiran for 1½ minutes, rinsed, and dried. To each hand equal amounts of a suspension of *Staphylococcus albus* were added, and allowed to dry for 10 minutes.	
Count on right hand (control)	6,520,000
Count on left hand (treated)	20,000

Similar results have been obtained with other microörganisms, *e.g.*, *Proteus vulgaris*, *Streptococcus viridans*, *N. catarrhalis*

It is known that the usual preoperative surgical brush-up, *e.g.*, 10 minutes with soap, followed by a brief dip in alcohol, falls far short of completely degerming the hands. We

§ Most of our experiments have been performed with Phemerol and Zephiran, but we have performed experiments with the following cationic detergents, which give essentially the same results: Squibb MC-474, Squibb MC-489, Squibb MC-507, Emulsol 605, Emulsol 607, cetyl pyridinium chloride, Damol, and Catol.

‖ These results on "apparent sterilization" show the need of using a specific neutralizing agent such as soap in all bactericidal studies with the cationic detergents. This precaution was stressed by Neufeld, Schiemann, and Schutz (*Z. f. Hyg. u. Infektionskr.*, 1940, **123**, 147). These authors also recognized that Zephiran was strongly adsorbed on human skin, and could kill pneumococci placed on a Zephiran-treated hand. However, they appear to have overlooked the protective nature of the adsorbed Zephiran film against release of the underlying micro-organisms.

have obtained the same results, but have found that an additional dip for a few minutes in one percent Phemerol or Zephiran protects the hands under surgical gloves during operations lasting several hours. That is, a 90-second scrub in water at the end of the operation fails to release organisms from the hand previously protected by the detergent film, whereas numerous organisms are removed from the control untreated hand. It has been found, also, that there is no significant multiplication of organisms under the detergent film during the course of prolonged surgical operations.

The film resists ordinary mechanical trauma, as well as brief dips in water or blood, and also exposure to animal tissues, but it is disintegrated by continuous exposure to running tap water for 15 to 30 seconds. Continuous dips in water or physiological saline also disintegrate it. Chemical substances like soap and strong alcohol break the film.

Further evidence for the presence of a film of cationic detergent on the skin may be obtained by dipping one hand in a one per cent solution of the detergent, and the other hand in water, and drying both. If both hands are then dipped in a saturated solution of an anionic dyestuff such as Guinea Green B or Rosindulin 2B for a few seconds and then rinsed in water, it will be found that only the hand which had been dipped in the detergent will be stained by the dyestuff. The influence of wetting action *per se* has been eliminated; it was found that thoroughly cleansed hands which can be wetted by the dyes are not stained by them unless a cationic detergent has been applied previously.

We have done some experiments on individuals performing abdominal operations on animals. The operators worked without gloves, and had one hand covered with the detergent film, whereas the other served as the untreated control. In almost every instance, despite the fact that the operators' hands became quite bloody in the course of several hours manipulation, there was a striking reduction in the number of organisms obtained from the treated hand upon a 90-second brush-up in water as compared with the control hand. However, it should be mentioned that in those instances when the operators were forced to dip their hands frequently in saline solutions, or to use many wet sponges, the detergent film showed signs of considerable disintegration.

Most of the cationic detergent germicides have been applied heretofore to human skin in concentrations of 0.1 per cent. We have not observed any signs of local or systemic effects from the use of one per cent solutions of Phemerol or Zephiran in single experiments on over 100 individuals. We have also studied the effects of one per cent Phemerol solution when applied frequently to the hands and have found that it can be tolerated without local or systemic toxicity when applied as a brush-up or rinse for 5 to 15 minutes each day over a period of six weeks.¶

There are hundreds of cationic detergents which have been synthesized, and many of these are available commercially. One of the reasons we wish to present this paper is to encourage others to search for suitable detergents of low toxicity, and also for a detergent which will deposit a film with greater resistance to solution in water.

It is believed that these results point the way for improved methods of hand hygiene, *e.g.*, for surgeons, medical personnel dealing with contagious cases, food-handlers, etc. In instances when surgical gloves are not available, it might be feasible to perform a reasonably sterile operation by protecting the hands with frequent dips in a solution of one percent cationic soap.

Summary. Certain cationic soaps deposit an invisible, non-perceptible film on the hands. This film retains bacteria underneath it, and is very resistant to mechanical trauma. The inner surface of the film has a low bactericidal power, whereas the outer surface exerts a strong germicidal action.

¶ Ten medical students participated in this study. Nine of them tolerated the use of the solution perfectly well; one developed a small area of hyperkeratosis which may have been partly due to mechanical trauma.

14355

Specific Stimulators of Hematopoiesis from Beef Liver.

D. L. Turner and F. R. Miller.
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In recent papers, we described the biological action,[1] and some of the chemical properties[2] of two substances found in the urine of patients with various diseases of the leucemia group; fractions from urine containing these substances, when injected into guinea pigs, produced specific myeloid or lymphoid proliferation in the organs of the animals. A theory was proposed to explain the biological significance of the two substances;[1] this theory included the assumption that the two specific materials were products of normal metabolism. We have tested this assumption by investigating normal beef liver as a source of the two substances.

The present work was designed primarily to determine whether the two stimulating factors occurred in normal organs. For this reason, the extraction method that proved successful in the case of urine[2] was used again. The separation of the active material from impurities was not quite as complete as with the urine extracts; thus, the lead salts soluble in ether retained activity, as shown in Table I. It was possible, however, to demonstrate the presence of a mixture of the two stimulating factors in the lipids of beef liver. Such a mixture, when injected into guinea pigs, gave a biological response resembling Hodgkin's disease.[1,2] The mixture was separated into two fractions producing proliferation of either the myeloid or lymphoid cells respectively; the two fractions were hydroxy-acids (lymphoid) and non-carbinol acids (myeloid).[2] This separation is illustrated by the results shown in Table II.

The fraction from urine that stimulated the proliferation of myeloid cells was converted previously[2] by catalytic hydrogenation to material that stimulated lymphoid proliferation; the reverse change was accomplished by oxidation. These changes have been effected with similar fractions from beef liver (Table II). The similarity in chemical properties illustrated by these reactions indicates that the factors of urine and liver that produce myeloid proliferation are identical; this applies also to the factors producing lymphoid proliferation. The major result of this work lies in the acquisition of an unlimited source of material for further study. The isolation of the stimulators in pure form from the urine of patients with leucemia seems difficult or impossible because of the limited supply of urine available.

Separation of Active Fractions from Beef Liver. Fresh beef liver was ground and dried; the dry material was thoroughly extracted with ethanol. The residue remaining after the distillation of the ethanol was leached with water until the water extracts were colorless. A portion of the residue, weighing 2 kg and corresponding to 43 kg of liver, was dissolved in 3 l. 95% ethanol. A solution of 600 g potassium hydroxide in 3 l. 95% ethanol was added. The mixture was heated to reflux for 4 hours. It was diluted with 3 l. ice water and extracted with petroleum ether (b.p. 30-60 degrees); this removed the neutral material soluble in petroleum ether. The aqueous solution was acidified with hydrochloric acid and the mixture was extracted with petroleum ether to obtain a fraction containing acid and phenolic material. The residue (A) from the evaporation of the petroleum ether was purified further by the same methods that proved effective with urine extracts,[2] except that the partition between petroleum ether and aqueous methanol[2] was omitted. The letters used in the previous paper[2] are used again in Table II to denote similar fractions. Fraction B from beef liver was esterified with a solution of dry hydrogen chloride in methanol;[3] after

[1] Miller, F. R., and Turner, D. L., *Am. J. Med. Sci.*, 1943, **206**, 146.

[2] Turner, D. L., and Miller, F. R., *J. Biol. Chem.*, 1943, **147**, 573.

[3] Pechmann, H., *Ann. der Chem.*, 1891, **261**, 159.

TABLE I.
Experiments with Discarded Fractions from the Purification.

Material	No. of animals	Dose, g	Dose as crude liver (calculated)	Biological reaction
Acids forming ether-soluble	3	0.6-1.4	220-510 g	0
lead salts	3	2.4-3.0	1000-1300 g	0 to (++) H
Material from aqueous filtrate of lead salts	4	0.2-0.4	15-20 kg	0
Neutral fraction	2	2.5	1200 g	0

TABLE II.
Separation and Interconversion of the Two Active Substances.

Material	No. of animals	Dose, g	Dose as crude liver, g (calculated)	Biological* reaction
B (acids)	6	0.6-1.8	260-780	(++) to (+++) H
C (hydroxy-acids)	6	0.09-0.16	740-1200	(+) to (+++) L
D (non-carbinol acids as methyl esters	5	1.1-3.5	650-2000	(++) to (+++) M
E (reduced D)	5	0.8-1.8	400-900	(+) to (+++) L
F (oxidized C)	6	0.1-0.2	560-1100	(+) to (+++) M

* H represents lesions in the animals that resemble the lesions of Hodgkin's disease; M and L similarly represent lesions of the myeloid and lymphoid type respectively.

acidic material had been removed from the product by extraction of its ethereal solution with sodium carbonate solution, the esters were separated into carbinols and non-carbinols in the usual manner.[4]

Interconversion of Active Fractions from Beef Liver. The non-carbinol esters (D) were hydrogenated in ether using Adams catalyst. This procedure followed that used for the corresponding fraction from urine;[2] the product was designated "E."

Hydroxy-acids (C) were oxidized with permanganate as described previously.[2] This product was designated "F."

Administration of the Fractions to Animals. Guinea pigs were given the material in 3-4 equal doses at 4-day intervals. The total dose is shown in the accompanying tables. In the tables, the numbers in the column representing the total dose in terms of fresh liver were calculated by assuming that the total weight of each fraction represented the quantity of raw material used in the preparation. Possible losses of active material in the fractionation, and the possibility that the active material was distributed through the fractions without a sharp separation should be considered in evaluating the significance of these numbers.

The material was injected subcutaneously or intramuscularly. The larger doses were given undiluted; the smaller in sesame oil. The guinea pigs were killed when they became moribund; this occurred 3 to 6 weeks after the first dose was given; sections of lungs, liver, spleen, lymph nodes, kidney, adrenal, and bone marrow were taken for histological examination.

The details of the experiments are summarized in the accompanying tables.

Nature of the Induced Lesions. The lesions produced in the guinea pigs given material B were the same as those described elsewhere;[1] the latter occurred in guinea pigs given a similar fraction from the urine of patients with Hodgkin's disease or monocytic leucemia. These lesions are identified by the letter "H" in the tables; the purely myeloid or lymphoid lesions are designated "M" and "L" respectively; they have been described previously.[1] The intensity of the biological response is roughly indicated by the repetition of (+) signs.[2]

Summary. The two substances that stimu-

[4] Marker, R. E., *J. Am. Chem. Soc.*, 1938, **60**, 2442.

late hematopoiesis, previously found in the urine of patients with diseases of the leucemia group,[1,2] have been shown to occur in the acid fraction of beef liver lipids.

14356

Electrophoretic Patterns of Seminal Plasma from Some "Abnormal" Human Semens.*

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We have previously published[1] some chemical and electrophoretic data on the proteins of normal human seminal plasma. We have since had an opportunity of obtaining semen specimens which were considered "abnormal" in one respect or another. These have been examined electrophoretically and, to a limited extent, chemically as well, to learn whether any differences between the proteins of such specimens and normal ones could be detected. The patterns are recorded here together with several additional ones of normal seminal plasmas.

The earlier work had demonstrated the presence in human seminal plasma of the following electrophoretic components: P1, the proteose fraction ($\mu = -.6 \times 10^{-5}$ cm^2 volt^{-1} sec^{-1}); P2 and P3, globulins,† each with a water soluble and a water insoluble fraction ($\mu = -2.9$ and -4.6 respectively), and probably a third globulin (P2a) with an intermediate mobility. A glycoprotein P4 ($\mu = -5.7$) is also present as well as a still faster moving component P5 ($\mu = -6.3$), which was considered related to P4 and possibly derived from it.‡ P4 and P5 are not always present together in the same specimen.

Methods and Results. The methods employed were as previously described.[1] All electrophoretic examinations were done on cell free, centrifuged specimens in phosphate buffer containing 0.055 M NaCl (pH 7.85, ionic strength 0.1) at ca. 2°C.

The electrophoretic patterns 1, 2, and 3 (Fig. 1) are of 3 normal specimens and show the presence of P1 (proteoses), P2 and P3 (globulins), and either P4 or P5 or both (glycoprotein). P2a was present in one of the 3 specimens and in the pooled mixture of 5 normal specimens, as is shown in pattern 4.

The usual peaks P1, P2, P3, and P5 were seen in the patterns, shown in illustrations 5 and 6 in the figure, of 2 abnormally viscous

* This work was made possible by a grant from the Committee on Maternal Health, Inc., which is gratefully acknowledged.

1 Ross, V., Moore, D. H., and Miller, E. G., Jr., *J. Biol. Chem.*, 1942, **144**, 667.

† We refer to P2, P2a, and P3 as globulins because there are water soluble and water insoluble fractions of each of these in fresh seminal plasma. If, however, seminal plasma is allowed to stand for several days before dialysis against water, no insoluble fraction separates. The term "globulin" may, therefore, not be accurate.

‡ When acetic acid is added to the water soluble fraction following dialysis of a fresh specimen against water, the precipitate is stringy and is soluble in 0.1% acetic acid containing 1% sodium chloride. If the acetic acid is added to the fresh, undialyzed plasma, the precipitate is not stringy and does not dissolve in this solution. When these operations were carried out on separate portions of the 5 pooled specimens (pattern 4, Fig. 1), the mobilities of the precipitates, following purification by dissolving in sodium hydroxide and reprecipitation with acetic acid, were 6.0 and 7.0 respectively (ascending, 1 hour). The two kinds of acetic acid precipitate, on analysis, yield similar values for nitrogen and reducing substance following hydrolysis.[1] Absorption in the ultraviolet[2] by the two forms is similar also.

2 Ross, V., and Ross, L., in press.

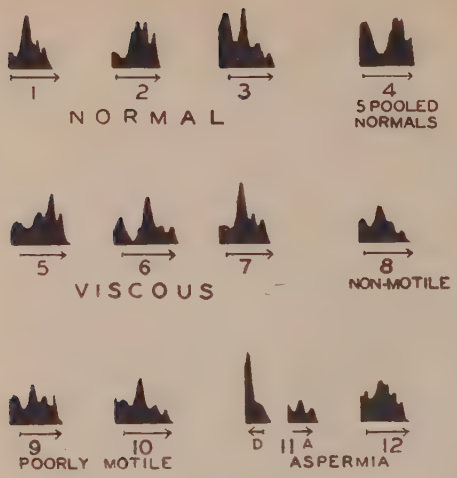

FIG. 1.

Electrophoretic patterns of seminal plasma from normal and "abnormal" human semens. Numbers under the individual patterns refer to the specimens listed in Table I. All pictures are of the ascending limb except in Pattern 11 where, because the light was filtered out in the region between components P2 and P3, portions of the ascending and descending limbs are shown.

specimens which failed to liquefy in several hours. One of these specimens had, in addition, component P2a. A sample from the same individual at another time also contained P2a, but this time lacked P2 (pattern 7). P4 was not observed in any of the 3 plasmas. This may possibly be related to the failure to liquefy normally. The addition of acetic acid to a portion of the specimen illustrated in pattern 6, yielded a precipitate which on resolution had an electrophoretic mobility of 6.2, while the pattern of the supernatant fluid contained no peak with a similar mobility. This happens in normal seminal plasmas also, and rules out the possibility that the peak with this mobility represents albumin.

The plasmas from the group of semens in which the sperm were either poorly motile or non-motile, or in which no sperm were present, contained the proteins P1, P2, P3, and P5 (patterns 8, 9, 10, 11, and 12). P2a was also present in one of the 2 aspermic specimens and possibly in the other, since there was in the specimen of plasma shown in Fig. 11, some component which filtered out visible and infra-red light and had a mobility between those of components P2 and P3. The descending limb of the electrophoresis cell gave evidence of the presence of P4 in the specimen represented by pattern 8.

Protein concentrations of several of the specimens were determined from the electro-

TABLE I.

Electrophoretic Mobilities of Proteins in Seminal Plasma from Normal Semen Specimens and from Some "Abnormal" Semen Specimens.

Phosphate buffer containing 0.055 M NaCl, pH 7.85, ionic strength = 0.1, Potential = 6.3 volts per cm.

Pattern No.	Kind of specimen	Mobilities × 10^5					
		P1	P2	P2a	P3	P4	P5
1	Normal	0.7	3.2		4.8	5.8	
2	" *	0.2	2.9	3.8	4.4		6.4
3	"	0.0	2.4		4.5	5.4	6.1
4	5 pooled normals	1.1	2.6	4.2	4.9	5.6	
5	Viscous	†	3.1		5.0		6.3
6‡	"	0.3	2.6	3.6	5.2		6.4
7‡	"	0.2		3.4	5.1		6.5
8	Non-motile sperm*	0.2	2.8		4.8		6.7
9	Consistent poorly motile sperm	0.1	2.9		4.8		6.4
10	Poor motility, low count	0.4	2.9		5.1		6.6
11	No sperm present§	0.8	2.6		4.8		6.0
12	" " "	0.9	3.1	4.1	4.9		6.3

Mobilities were calculated from 1½-hour ascending patterns except in Pattern 11 in which both ascending and descending patterns were used and Pattern 6 in which the 2-hour pattern was used.

* P4 peak was present in descending limb pattern.

† P1 had a slight positive charge in this experiment.

‡ Two specimens from the same individual.

§ P2a might have been present also. The light was filtered out by the solution in the cell in the region where P2a would be seen if present.

phoretic patterns. The values were: for pattern 1, 0.7%; pattern 7, 0.7%; pattern 8, 0.7%; pattern 9, 0.8%; pattern 10, 0.8%. Thus there was no significant difference, in this respect, between the plasmas of several kinds of abnormal semens and that of a normal specimen. The refractive indices of the several protein components were assumed to be the same and equal to that of serum globulin.

Summary. Electrophoretic examination of the plasmas from specimens of human semen which were either (1) abnormally viscous or in which (2) the sperm were either poorly motile or non-motile, or which (3) contained no sperm revealed no definitely significant deviation in protein components from normal specimens. Components P1 (proteose), P2 and P3 (globulins) were present in all these specimens as they are in normal ones. Component P2a (globulin), described in an earlier report as being present in occasional normal specimens, was found in some of the abnormal ones also. Normal specimens contain either P4 or P5 (glycoprotein) or both. Component P5 was present in all the abnormal specimens. The absence of P4 from all but one of these is noteworthy, but the varied nature of the abnormality makes interpretation difficult.§

§ We wish to record our appreciation to Dr. John MacLeod and Dr. Robert S. Hotchkiss of Cornell University Medical College for the semen specimens.

14357

The Cold Agglutination Test in the Diagnosis of Primary Atypical Pneumonia.*

GORDON MEIKLEJOHN. (Introduced by Monroe D. Eaton.)

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Since February, 1943, when Peterson, Ham, and Finland[1] reported on cold autohemagglutination in primary atypical pneumonia and suggested that this procedure might differentiate the primary atypical pneumonias from other types of pneumonia, all serum specimens coming to this laboratory from patients with respiratory diseases have been tested for cold agglutinins. The chief purposes of this investigation have been (1) to determine how frequently significant titers of cold agglutinins are found in primary atypical pneumonia and (2) to determine how often similar titers are encountered in other respiratory diseases.

Materials and Method. 155 serum specimens from 74 patients diagnosed as primary atypical pneumonia were tested. These came from 3 hospitals where the disease is common. Diagnosis was made on the basis of the clinical picture, sputum examination, and X-ray findings. Three or more specimens were obtained from each of 20 patients, 2 specimens from 34 patients, and one specimen from 20 patients.

The control group consisted of 133 serum specimens from individuals with the following diagnoses: Bacterial pneumonias (mostly pneumococcal) 22, pulmonary tuberculosis 23, febrile upper respiratory infections 17, influenza (type A), 7, coccidioidomycosis 6,† pneumonias of uncertain etiology 8, lymphogranuloma venereum 20, and normal individuals 36. All specimens in this group were taken later than the 10th day of disease and were tested while fresh.

The technic employed was as follows: Twofold serial serum dilutions commencing at

* The studies and observations on which this paper is based were conducted with the support and under the auspices of the International Health Division of the Rockefeller Foundation in cooperation with the California State Department of Public Health.

1 Peterson, O. L., Ham, T. H., and Finland, M., *Science*, 1943, **97**, 167.

† These specimens were supplied by Dr. C. E. Smith, Stanford Medical School.

TABLE I.
Distribution of Highest Titers Obtained by Each Individual Tested.

	Highest titer									
	<10	10	20	40	80	160	320	640	1280	2560
Primary atypical pneumonia	12	2	15	9	10	9	7	6	3	1
Control group	133	4	1*	0	0	0	0	0	0	0

* Does not include 3 specimens with titers of 20 from (1) 2 patients with pneumococcal pneumonia on third day and (2) a patient with streptococcal pharyngitis on fourth day of disease.

dilutions of 1:5 or 1:10 were made in normal saline. To 1.0 cc of diluted serum was added 1.0 cc of washed type O human cells. The mixture was thoroughly shaken and placed in the icebox at a temperature between 0° and 4°C for one hour. Coarse agglutination and accelerated sedimentation were interpreted as a positive test. Following reading, the suspensions were allowed to come to room temperature or, in the event that titers were unusually high, to 37°C in order to ascertain that the agglutination was reversible at higher temperatures. Titers were expressed in terms of the original serum dilution, *i.e.*, before addition of an equivalent volume of cell suspension.

Results in Control Group. One patient classified as having an upper respiratory infection showed a rise in titer during convalescence from less than 10 to 20. Two patients with pneumococcal pneumonia showed a titer of 20 on the third day of illness. Three patients with pulmonary tuberculosis and one with a bacterial pneumonia had titers of 10. All other sera had titers of less than 10.

On the basis of these results it seemed reasonable to consider titers of 20 to be probably significant and titers of 40 or more to be definitely beyond the normal range.

Results in Primary Atypical Pneumonia. Table I summarizes the results according to the highest titer reached by each individual tested. Sixty of the 74 patients diagnosed clinically as primary atypical pneumonia had titers of 20 or more in at least one specimen. Forty-five had titers of 40 or more. Thus approximately three-fifths had titers clearly beyond normal limits, one-fifth had titers which were probably significant, while the remaining one-fifth failed to show significant titers. It is worthy of mention that sera from 26 of those individuals who subsequently showed titers above 20 were negative when tested early in the course of the disease. Significant titers were observed more frequently in those individuals who were tested repeatedly.

The height of the titer attained showed great individual variation. Although a number of individuals tested once, and a few tested several times, failed to develop titers above 20, the majority had titers in the range between 40 and 640. The highest titer observed was 2560. The severity of the illness did not appear to have a direct relationship to the height of the titer.

It soon became clear that the time at which a specimen was taken was of considerable importance. In Table II the titers of the 130 specimens obtained from those 60 patients who at some time during illness or convalescence had titers of 20 or more are arranged according to the period of disease at which the specimen was taken. During the first week of disease less than one fifth of the sera tested showed significant titers. Rise in titer usually began between the eighth and tenth days, with the titer reaching a peak between the twelfth and twenty-fifth days and falling off fairly rapidly after the thirtieth day.

Only 2 patients with pneumonia due to psittacosis-like viruses, proven in both cases by isolation of virus from sputum, have been tested. One showed a titer of less than 10 on the eighth day of disease. Serum specimens from the other showed titers of 10, 10, 20, and 10 respectively on the sixth, tenth, fifteenth, and thirty-first days of disease. In the latter case complement-fixing antibody to lympho-

TABLE II.
Relationship of Titer to Stage of Disease of 130 Serum Specimens from 60 Patients Who at Some Time Showed Titers of 20 or More.

Titer	Day of disease specimen collected				
	Less than 7	8-10	11-20	21-30	More than 31
<20	22	4	2	2	4
>20	4	9	53	23	7

granuloma venereum antigen[2] had risen during this period from less than 6 to more than 24. Twenty-six other sera, positive by cold agglutination, failed to show a significant rise in complement-fixing antibody to the meningopneumonitis virus.[2]

Observations on Certain Properties of Cold Agglutinins. Cold agglutinins are readily adsorbable by red blood cells at low temperatures. For this reason tests are most satisfactorily done with sera separated at temperatures above 20°C. Separation of serum at low temperatures causes sufficient drop in titer to cause sera in the lower titer range (40 or less) to fall below the level considered significant. With high titer sera this is of less importance. The titer of a serum is not changed by heating at 56°C for one-half hour, but is diminished by heating at 60° and destroyed at 65°C.

Storage of serum at 4°C results in a gradual falling off in titer. Twenty specimens were retested after storage for 2 to 5 months. Ten of the 11 specimens which had shown titers of 20 or 40 when fresh had dropped to 10 or less. The 9 specimens which had shown titers in excess of 80 showed a fall in titer, but 8 of the 9 were still above 20. Two sera, stored for 10 and 14 months respectively, still showed titers of 80.

The thermal range of activity of the agglutinations varied with the titer. Two sera, with titers of 1,280 and 2,560 showed agglutination up to 21°C, but not at 25°C. This was of interest because one of these specimens had come from a patient who, after recovering from a comparatively mild primary atypical pneumonia, had died suddenly 17 days after onset as a result of pulmonary embolism.

Discussion. Progress in the field of the primary atypical pneumonias has been retarded by the fact that the etiology remains obscure. No laboratory procedure has been available by means of which the great majority of these pneumonias can be classified. In the absence of a specific etiological test the cold agglutination test may offer a roundabout way of attaining this objective. Data presented in other reports[3,4] and in this paper suggest that a large proportion of cases develop significant titers of cold agglutinins. It is not yet clear whether those cases which fail to develop cold agglutinins represent the same disease or belong in other etiological groups.

To the clinician the value of the test in the ordinary case of primary atypical pneumonia lies in the fact that it offers an objective means of confirming a diagnosis which can usually be made on the basis of the clinical picture, sputum examination, and x-ray findings The fact that cold agglutinins seldom appear in significant titer until the second week of disease means that in most instances they appear only after the patient is on the road to recovery. In certain types of cases, notably the protracted case in which diagnosis is uncertain, the rare cases of mixed "virus" and bacterial etiology, and in those in which tuberculosis or coccidioidomycosis is suspected, the test may prove especially useful.

Transmission of an infectious agent from patients with primary atypical pneumonia to cotton rats has been reported in a previous communication.[5] It is of interest that this transmission appears to be successful far

[2] Eaton, M. D., and Corey, M., PROC. SOC. EXP. BIOL. AND MED., 1942, **51**, 165.

[3] Turner, J. C., Nisnewitz, S., Jackson, E. B., and Berney, R., *Lancet*, 1943, **21**, 765.

[4] Horstman, D., and Tatlock, H., *J. A. M. A.*, 1943, **122**, 369.

[5] Eaton, M. D., Meiklejohn, G., Van Herick, W., and Talbot, J. C., *Science*, 1942, **96**, 518.

more frequently in those cases which develop cold agglutinins than in those which do not. Studies on this point are in progress.

Summary. Significant titers of cold agglutinins were demonstrated in a large proportion of cases of primary atypical pneumonia. Similar titers were not observed in sera from patients with a number of other respiratory diseases.

The author wishes to acknowledge the assistance of the Medical Staff of the University of California Hospital and of the visiting and laboratory staffs of Cowell Memorial Hospital.

14358

On the Mechanism of Fever Production with Inflammation.*

VALY MENKIN.

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Recent studies undertaken by the writer have demonstrated the presence of a substance in exudates which offers a reasonable explanation for the basic pattern of injury in inflammation.[1,2] This substance, which is either a euglobulin or is at least associated with that fraction of exudates, has been termed "necrosin." It is presumably liberated from the cell which has been initially injured by an irritant. The internal chemistry of the damaged cell is doubtless altered yielding as a result various common denominators, which in turn are responsible for the unfolding of a fundamentally stereo-pattern in inflammation. Leukotaxine, the leukocytosis-promoting factor. and necrosin belong to such a category of chemical units formed by the injured cells.[3] In this connection necrosin has been found to induce a severe inflammatory reaction accompanied by lymphatic blockade.[1,2] This substance introduced into the circulating blood is followed by a prompt leukopenia replaced several hours later by a leukocytosis.[2] The internal organs are injured; notably the liver and to some extent the kidneys.[2] Besides fatty deposits in the parenchyma of these structures, small foci of leukocytic infiltration may be found irregularly scattered throughout these organs.[2]

The present communication summarizes further data indicating that the intravascular injection of necrosin is accompanied by a rapid elevation in temperature. This hyperthermia is not induced by other protein fractions derived from either exudate, ascitic fluid, or normal blood serum. Inasmuch as necrosin seems to penetrate from the site of inflammation into the circulating blood,[2] it is conceivable that the basis of fever production with inflammation may be referable in large part to the absorption of necrosin from the site of injury into the blood stream. Owing to limit on space the earlier literature on fever will be omitted in the present short communication.

In the present experiments dogs were injected with necrosin either in the form of an aqueous suspension or as the desiccated material which was taken up in several cubic centimeters of physiological saline. The injections were made into the circulating blood by cardiac puncture. The rectal temperature was recorded at approximate hourly intervals. The chemical preparation of necrosin from canine exudates has been described in earlier

* The work described in this paper was done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and Harvard University. The studies were also aided in part by a grant from the Jane Coffin Childs Memorial Fund for Medical Research.

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† Present address, Fearing Research Laboratory, Free Hospital for Women, Brookline, Mass.

1 Menkin, Valy, *Science*, 1943, **97**, 165.

2 *Idem, Arch. Path.*, 1943, **36**, 269.

3 *Idem, Dynamics of Inflammation*, Macmillan Company, 1940.

TABLE I.
Effect of Necrosin or the Euglobulin Fraction of Exudate on the Temperature Level of Dogs.

Dog No.	Dose of necrosin injected into the circulating blood	Temperature prior to injection of necrosin, °F	Max. temp. level within about 5 hr after inj. of necrosin, °F	Increase in level of temp.
7-92	8 cc of necrosin suspension*	101.4	104.2	2.8
7-92	10 " " " "	101.6	105.3	3.7
7-91	10 " " " "	101.8	105.3	3.5
7-83	10 " " " "	100.9	103.7	2.8
7-40	10 " " " "	101.0	106.2	5.2
7-92	100 mg of desiccated necrosin taken in 10 cc saline	101.4	102.0	0.6
7-92	Same as above	101.0	104.1	3.1
7-94	About 75 mg of desiccated necrosin taken in 6 cc saline	101.1	104.1	3.0
7-75	50 mg of desiccated necrosin taken in 10 cc saline	102.2	104.4	2.2
Avg elevation in temp.				3.0

* Preserved on ice under layer of toluene.

TABLE II.
Effect of Various Protein Fractions Derived from Exudates (Except Necrosin), from Blood Serum, and from Ascitic Fluid, on the Temperature Level of Dogs.

Dog No.	Type and dose of material inj. into circulating blood	Temp. prior to inj. of material, °F	Max. temp. level within about 6 hr after inj. of material, °F	Change in temp. as result of inj. of material
7-80	40 cc of aqueous suspension of leukocytosis-promoting factor (pseudoglobulin of exudate)	101.0	101.4	+0.4
7-91	100 mg of desiccated leukocytosis-promoting factor (pseudoglobulin of exudate) in 10 cc saline	101.1	101.0	—0.1
7-94	142 mg of desiccated LPF (leukocytosis-promoting factor) in 10 cc saline	102.3	101.8	—0.5
7-91	15 cc of LPF in water	102.2	102.4	+0.2
7-92	20 cc of albumin solution derived from an exudate	101.5	101.7	+0.2
7-78	65 mg of euglobulin from ascitic fluid in 10 cc saline	103.2	102.3	—1.0
7-80	25 mg of euglobulin from normal blood serum in 10 cc saline	101.0	101.0	0

communications and will therefore not be repeated here.[1,2]

The results are tabulated in Table I. It is clear that the introduction into the circulation of necrosin is accompanied by an appreciable elevation in temperature, averaging three degrees (Fahrenheit). The rise occurs very promptly and is very definitely perceived within about an hour or two after injection of the material (Chart 1). In this connection it is to be noted that the injection of 20 cc of whole exudate in normal dogs induces a sharp and marked elevation in temperature. In contrast, the injection of normal blood serum fails to augment the temperature.

On the other hand, the euglobulin fraction of normal blood serum, the euglobulin derived from a sample of ascitic fluid from a human patient, several fractions of pseudo-globulin from canine exudates (*i.e.*, the leukocytosis-promoting factor), and finally the albumin fraction of exudates, have all failed to induce

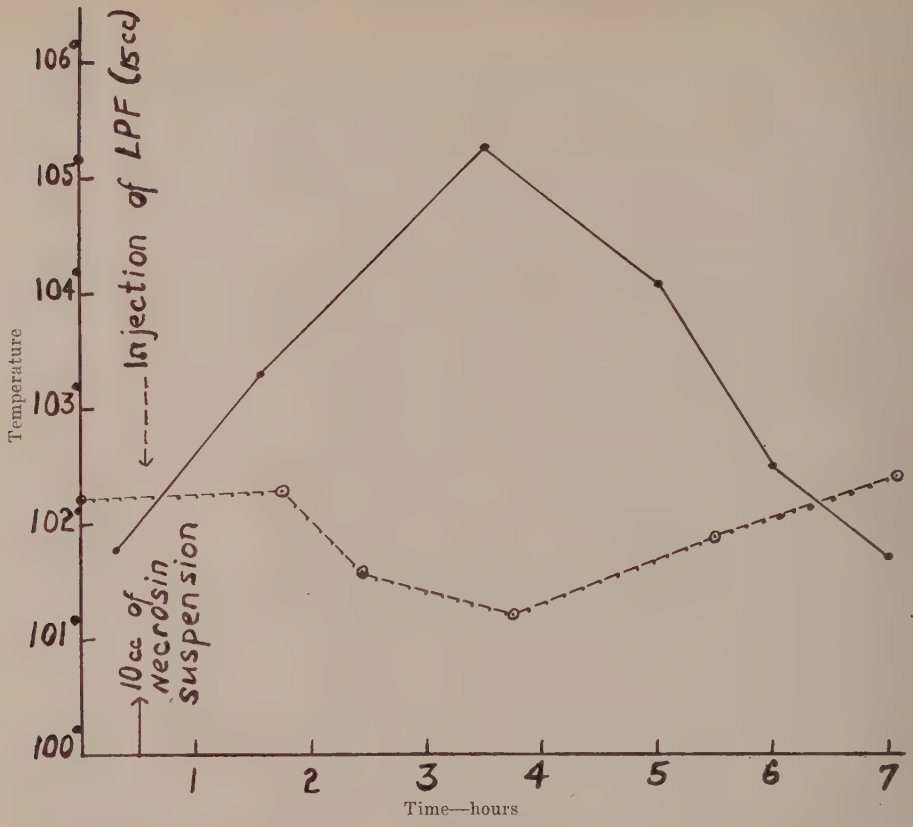

CHART 1.

The effect of necrosin and of the leukocytosis-promoting factor on the temperature level in the dog.

•————• Effect of necrosin on temperature level—Dog 7-91 (Degrees in Fahrenheit scale).

— — — •— — — Effect of the leukocytosis-promoting factor (LPF) on temperature—Dog 7-91 (Degrees in Fahrenheit scale).

any detectable fever in dogs (Table II, Chart 1). These facts indicate that evidently it is only the euglobulin fraction of exudates, *i.e.*, necrosin, which contains the active principle capable in turn of evoking an appreciable rise in temperature. Whether this fever production by necrosin occurs through the mediation of the heat centers remains to be determined. These studies are being pursued in an endeavor to elucidate further the mechanism involved in the development of fever with inflammation.

In conclusion, the observations presented indicate that necrosin, the euglobulin fraction recovered from exudates and which is *per se* primarily responsible for the basic pattern of injury in inflammation, in turn induces, contrary to other protein fractions of exudates, an appreciable degree of fever when injected into the circulating blood of dogs. The absorption of this substance into the blood stream from the site of an acute injury offers a reasonable explanation for the probable factor largely responsible in the development of fever with inflammation.

14359

Action of Bacterial Toxins on Tumors. VI. Protection Against Tumor Hemorrhage Following Heterologous Immunization.

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That active protection against tumor hemorrhage caused by injection of bacterial products can be induced by immunization with the homologous endotoxin has recently been demonstrated by us.[1] But that animals may be immunized with the endotoxin of one gram-negative bacterium and also exhibit a cross-immunity to the endotoxins of unrelated gram-negative organisms has, to our knowledge, not been previously reported.

During the course of this series of studies it has been observed occasionally that mice whose earlier history had included injections of the endotoxin of a gram-negative bacterium would, when used for work with implanted tumors, exhibit anomalous resistance to tumor hemorrhage following the injection of endotoxins of gram-negative species immunologically different from those used for the earlier injections. The present study represents an effort to test whether this anomalous protection against toxin-induced tumor hemorrhage is referrable to a cross-immunity mechanism.

Methods. (1) *Source of immunizing materials.* Three organisms, 2 closely related and the third taxonomically remote from the other two, were chosen for these experiments. They were *Shigella paradysenteriæ* Flexner, *Salmonella typhimurium* and *Rhodospirillum rubrum.* These organisms were grown in large quantities in suitable liquid culture media.[2] The cultures were killed by adding phenol to 2%, reduced in volume by pervaporation and precipitated from 66% acetone. Aqueous solutions of these precipitates, injected intraperitoneally, were used throughout the tests. Constant care was taken to prevent contamination, each culture flask being streaked on plates before killing to confirm its purity. The parent cultures were checked for purity by agglutination tests against standard typing sera. These and other precautions to prevent contaminations of cultures are described in detail elsewhere.[2]

(2) *Immunization.* Nine separate groups of white Rockland male mice weighing 20-22 g were subjected to the following schedule of immunization: ¼ LD50 was injected on the first day; ½ LD50 on the third day; 1 LD50 on the fifth day; 1 LD50 on the sixth day; and 2 LD50 on the eighth day. This schedule of immunization was employed for each of the 3 endotoxin preparations. During the course of this immunization between ten and thirty per cent of the animals were lost, presumably because of primary toxicity of the injected material.

(3) *Tumors.* On the eighth day of the immunization schedule bits of Mouse Sarcoma 180 tissue were implanted under the axillary skin according to a standardized trocar implantation technic.[3] The tumor implant was allowed to grow for 7 days, by which time it had become well vascularized and had developed into a spherical mass about 1 cm in diameter. At this time a test injection of endotoxin-containing material was made. Following injection of the endotoxin-containing materials, hemorrhage in similar tumors carried by normal (non-immunized) mice reaches its maximum at 5 to 8 hours. The experimental animals were usually sacrificed about 12 hours after injection and the tumors dissected. The degree of tumor hemorrhage was graded as slight (1), considerable (2), and marked (3). Normal tumor tissue is pink; hemorrhagic tumor tissue is deep red to deep purple.

Results and Discussion. Previous experiments[1] have indicated that in normal non-

[1] Zahl, Paul A., Hutner, S. H., and Cooper, F. S., PROC. SOC. EXP. BIOL. AND MED., 1943, **54**, 48.

[2] Zahl, Paul A., and Hutner, S. H., *Am. J. Hyg.*, in press.

[3] Hutner, S. H., and Zahl, Paul A., PROC. SOC. EXP. BIOL. AND MED., 1943, **52**, 364.

TABLE I.

Toxin-produced Tumor Hemorrhage in Homologously and Heterologously Immunized Mice.

Toxin used for immunization	Toxin used for tumor hemorrhage production	Amount injected and degree of hemorrhage*			
		1/40 LD50	1/4 LD50	1 LD50	4 LD50†
Shigella	*Shigella*	0 0	0 0 0	0 0 0	3 3 3
"	*Salmonella*	0 0 0 0 0	0 0 0 0 0 2 1 1 1 1	0 0 0 0 0 0 0 0 2 1 2 1 2	2 2 3 3 3 3
"	*Rhodospirillum*	0 0	0 0 0 0 0 0 0 1 2 1 2	0 0 0 0 1 2 2	0 0 1 1 1 2 2 3
Salmonella	Salmonella	0 0 0 0 0 0	0 0 0 0 0 0	0 0 0 0 0 0 0 0 1 1 2 2 2	0 0 2 2 2 2 2 2
"	*Shigella*	0 0	0 0 1 2		2 3
"	*Rhodospirillum*	0 0	0 0 1 1	0 0 0 2 2 1	3 3 3
Rhodospirillum	"	0 0 0 0	0 0 0 0	0 0 0 0 0 1 1 2 3	1 2 3
"	*Salmonella*	0 0 0 0	0 0 0 0 1 1 2 2	0 0 0 0 0 0 0 1 1 2 2 2 2 3	0 2 2 2 2 3 3 3
"	*Shigella*	0 0	1 3	2 3	3 3
Non-immunized	"	1 2 2 2 3 3 3 3	2 2 2 3 3 3	2 2 2 3 3	2 2 3 3 3 3
"	*Salmonella*	0 2 2 2 3 3 3 3 3 3 3	1 1 2 3 3	2 2 2 3 3 3 3	
"	*Rhodospirillum*		0 0 1 2 2 2	1 1 1 2 2 3 3 3	

* Each designation below represents one animal. 0 = no hemorrhage; 1 = slight hemorrhage; 2 = considerable hemorrhage; 3 = marked hemorrhage.

† Some of the mice indicated below succumbed to the lethal effects of the toxic material, but hemorrhage was evident before death.

The amount of dry toxic material necessary to produce death within 24 hours of 50% of non-immunized mice injected intraperitoneally with the aqueous solution was: *Shigella*, 8.0 mg; *Salmonella*, 2.2 mg; *Rhodospirillum*, 6.0 mg.

immunized animals, and using relatively unpurified preparations obtained from gram-negative organisms, 1/200 of an LD50 is sufficient to induce hemorrhage in tumors. It was found that tumor hemorrhage in immunized mice could not be induced regularly with a dosage lower than one LD50. To test degree of immunity in the animals of the present experiment, 4 arbitrary dosage levels were therefore selected: 1/40, 1/4, 1, and 4 LD50. In non-immunized animals these dosages of the 3 toxic materials gave regular and uniform hemorrhage (see Table I).* On the other hand, among the immunized animals, there was no tumor-hemorrhage whatever following injection with 1/40 LD50, very little with 1/4 LD50, some with 1 LD50, but uniform tumor hemorrhage occurred with 4 LD50. The results (Table I) indicate that cross-protection appears to exist at about the same level as the homologous protection for each of the 3 organisms.

Tumor hemorrhage is a sensitive indicator of the type of vascular damage produced by the endotoxins of gram-negative bacteria. It is generally considered that the lethal effect of these endotoxins results from a similar but generalized vascular damage.

* With the exception of *Rhodospirillum* which was not tested in a dose of 1/40 LD50 in controls.

Boivin and Mesrobeanu,[4] using lethality as an index, failed to obtain cross-protection with the purified toxic O antigens of species of *Pseudomonas, Shigella,* and *Salmonella.* We have repeated our cross-immunity experiments using lethality instead of tumor hemorrhage as a criterion, and we have observed definite cross-protection. A detailed discussion of this apparent contradiction, and the problems posed by the cross-protection reported among heterologous gram-negative organisms, is being published elsewhere.[2]

Our previous work with a large and highly heterogeneous assortment of gram-negative bacteria led to the postulation of the existence of a common toxic factor as the incitant of tumor hemorrhage among substantially all gram-negative bacteria.[3,5] The immunochemical work of Morgan and Partridge[6] and of the Boivin school had indicated that for the gram-negative bacteria studied, most of the toxicity is attributable to a portion of the O

[4] Boivin, A., and Mesrobeanu, L., *C. R. Soc. Biol.*, 1937, **125**, 273; 1938, **127**, 752; **128**, 835; *Rev. d'Immunol.*, 1938, **4**, 40; 1938, **4**, 469.

[5] Zahl, Paul A., Hutner, S. H., Spitz, S., Sugiura, K., and Cooper, F. S., *Am. J. Hyg.*, 1942, **36**, 224.

[6] Morgan, W. T. J., and Partridge, S. M., *Biochem. J.*, 1941, **35**, 1140; *Brit. J. Exp. Path.*, 1942, **23**, 151.

antigen, and that this portion is antigenic. This would lead one to anticipate the possibility of securing, under appropriate conditions, a relative cross-immunity to vascular damage with gram-negative organisms generally. The organisms used in the experiments reported here were selected as a test of this hypothesis, and the results observed support our earlier conclusions.

Summary. Mice immunized with endotoxin preparations of *Shigella paradysenteriæ* Flexner, *Salmonella typhimurium* and *Rhodospirillum rubrum* were found to have been protected against the induction of hemorrhage in implanted tumors.

Protection by immunization was found to be about as effective against the induction of tumor hemorrhage when the heterologous organisms were used as when the homologous were used. This finding supports our earlier hypothesis that a common antigenic toxic component is characteristic of gram-negative bacteria generally.

14360

A Rapid Method for Estimation of Penicillin

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In connection with chemical studies on penicillin and, more particularly, with efficient large scale production of the antibiotic substance, there has been an urgent need for a rapid method for the estimation of the substance. It had been hoped that the antibioluminescent activity of other antibiotic agents might be found to be a property of penicillin since use of this property gave a method for rapid titration of potency; such however did not prove to be the case.[1,2] Methods of titration requiring 3 to 6 hours delay are known and employed,[3], but with the rapid rise in activity seen in the more recent methods of penicillin production, this time interval is still too long for efficient production.

A report from England[4] stated that the group at the Wellcome Physiological Research Laboratories had devised a new method of testing for penicillin based on the hemolysis of red blood cells by hemolytic streptococci. This test was complete within 3 to 4 hours, *i.e.*, was not much faster than tests already in use. Rammelkamp[5] had already described an 18-hour test which was based on the hemolysis of red cells by hemolytic streptococci. In connection with studies on *Aspergillus flavus* in which the hemolytic streptococcus was used as the test organism[6] the present authors had occasion to note the very rapid appearance of hemolysis which occurred if conditions were optimal. This suggested that a method of titrating penicillin or other antibiotic substances could be devised with conditions so arranged that a reading could be made in as little as 60 to 90 minutes.

Of the strains of hemolytic streptococci tested, strain C-203 has produced detectable amounts of hemolysin most rapidly. Up to the present time no other group of bacteria has proven more satisfactory than *Streptococcus pyogenes*. Since penicillin acts only by

1 Rake, G., McKee, C. M., and Jones, H., PROC. SOC. EXP. BIOL. AND MED., 1942, **51**, 273.

2 Rake, G., Jones, H., and McKee, C. M., PROC. SOC. EXP. BIOL. AND MED., 1943, **52**, 136.

3 McKee, C. M., unpublished data; Foster, J. W., and Woodruff, H. B., *J. Bact.*, 1943, **46**, 187; Foster, J. W., and Wilker, B. L., *J. Bact.*, 1943, **46**, 377.

4 Wilson, U., *Nature*, 1943, **152**, 475.

5 Rammelkamp, C. H., PROC. SOC. EXP. BIOL. AND MED., 1942, **51**, 95.

6 Jones, H., Rake, G., and Hamre, D. M., *J. Bact.*, 1943, **45**, 461.

preventing growth of the cocci and has no action on hemolysin already produced, the first problem was to avoid the presence of preformed hemolysin which would act in the presence even of high concentration of penicillin. It has not proven possible to grow the organisms in fluid medium which will allow good growth without the production of hemolysin. In any medium in which the organism grew well, either plentiful hemolysin was produced or the organism developed variants which failed to produce hemolysin even in the presence of red blood cells. Moreover, because the hemolysin is apparently closely adsorbed on the surface of the organisms, it was not possible to remove it from them by throwing them down in a centrifuge and washing them repeatedly. The technic finally adopted was one of using cultures with so little preformed hemolysin as to be insignificant in the test. Cultures for inoculation are taken preferably during the logarithmic phase of their growth. The exact technic is given below.

Optimal conditions for the test have been determined. Beef heart infusion broth has proven the best medium; for red blood cells, 1% defibrinated rabbit's blood is better than any other concentration of the same or other blood; for incubation, most satisfactory results have been obtained by use of a water-bath at 37°C.

In performing the test a 2- to 3-hour culture in 1% rabbit's blood broth of C-203 (*i.e.*, one in which hemolysis is just appearing) subsequently stored overnight at 0°C, is used for the inoculation of a lot of 1% rabbit's blood broth in volumes of 0.1 ml of inoculum for each 2.0 ml of blood broth. As soon as this fresh culture shows detectable hemolysis (*i.e.*, within 2 hours), and the organisms are dividing rapidly, it is ready for use in the test. With such a culture, readings of potency can be read in 55 to 90 minutes. If it is desirable to test filtrates or other preparations without this 2-hour delay, the overnight culture may be used directly, but in this case it may be 2 hours before detectable hemolysin will be formed.

In carrying out the test, 2 ml amounts of 1% rabbit's blood beef heart infusion broth are placed in a series of 13 x 100 mm tubes. To each tube is added 0.1 ml of the appropriate dilution of penicillin standard or unknown filtrate. As a control of culture one tube receives 0.1 ml of distilled water. As a control of preformed hemolysin one tube receives 0.7 Florey units in 0.1 ml, *i.e.*, an amount of penicillin which will certainly inhibit growth of the streptococci. To each tube is now added 0.2 ml of culture. Contents of tubes are well mixed before incubation.

With the test as set out above, between .03 and .05 of a Florey unit in a total volume of 2.3 ml will prevent growth and production of hemolysin. In the standard the range used is from 0.08 to 0.01 Florey units (.08, .06, .04, .03, .02, .015, and .01). Filtrates are diluted and tested, according to their expected potency, in the following manner: 1/2, 1/3, 1/4, 1/6, 1/8, etc., up to 1/256.

In reading the test it has been found that the first signs of hemolysis can be most readily detected by whirling the tubes slightly and throwing a plume of red cells into the clear supernatant broth. In the presence of a trace of hemolysin this plume becomes rapidly hemolyzed. All tubes showing such commencing hemolysin are thoroughly mixed and replaced in the water-bath for 2 or 3 minutes by which time uniform hemolysis will have appeared. In most cases all tubes which are to show hemolysis will do so at almost the same time, and an end-point is obtained by taking the last tube in each series showing no hemolysis. Occasionally a tube shows hemolysis delayed by about 30 minutes over the others. Such a tube is given a ± reading and an interpolated potency.

The test can be set up rapidly and without sterile precautions. It can be performed with turbid or heavily contaminated samples. Potencies obtained by its use have been compared on many occasions with those obtained by more accurate 18-hour tests[7] and a remarkably good correlation has been obtained.

Summary. A rapid test for the estimation of penicillin or other antibiotic agents makes use of the hemolytic property of β streptococci. With optimal conditions, as set out above, results can be read within 55 to 90 minutes.

[7] McKee, C. M., Rake, G., and Menzel, A. E. O., to be published.

14361

Effect of Biotin on Chick Spinal Ganglia in Tissue Culture.*

AGNES S. BURT. (Introduced by Paul Weiss.)

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Hamilton and Plotz[1] have recently reported that biotin in concentrations of 1/6 to 5/6 γ per cc in tissue culture media markedly improves growth of embryonic chick and mouse explants, particularly those containing nerve cells. As their work appears to have been done on embryo fragments containing several different types of tissue, and on brain, nerve growth from which is very difficult to measure because of the fineness of processes and the influence of the liquefaction which frequently occurs in such cultures,[2] their experiments have been repeated using isolated spinal ganglia of embryonic chicks with the purpose of appraising biotin as a possible stimulant of axon regeneration. Spinal ganglia, previously used as test material in this laboratory,[3] produce abundant growth of axons, easily demonstrable in fixed material by silver stains, as well as of spindle cells and macrophages.

One hundred and eight spinal ganglia from the lumbo-sacral and thoracic regions of 9- to 13-day-old embryos were explanted into chicken plasma and embryo extract. In 55 cases, Biotin Concentrate No. 1000 (S. M. A. Corporation), furnished in 50% ethanol, was added to the clot in modified Tyrode's solution so that the final amount of biotin added to the medium was 1/3 γ per cc (the optimum stimulating concentration according to Hamilton and Plotz); and in 53 control cases, Tyrode's solution containing a volume of 50% alcohol equivalent to the volume of the biotin concentrate was added. The pH of the Tyrode's solution was adjusted to 7.4 with $NaHCO_3$ before it was added to the cultures in all cases. Ganglia from one side of any given embryo were used for controls and those from the other side for biotin treatment, but no attempt was made to pair cultures because individual differences among ganglia due to treatment during removal and, apparently, to inherent factors preclude the use of this method of comparison.

Observations were made on the living cultures at 24-hour intervals, and all cultures were fixed after 2 days, as previous experience has shown that maximum axon growth is obtained with this technic on the second day of incubation and that regression of nerve fibers begins soon after. Preparations were stained by Bodian's protargol method and density of nerve fibers, spindle cells, and macrophages was recorded by an arbitrary system in which Class I signified very sparse growth, Class II moderate growth, Class III dense growth, and Class IV very dense, almost solid growth. Standards of nerve fiber density have been illustrated in a previous publication.[3]

The 108 cultures were explanted in 5 groups, each containing control and treated cultures. In only one of these groups the biotin-treated cultures grew better than the controls, while in the other 4 groups biotin treatment either had no effect on growth of the various cell components or appeared to be slightly inhibitory. Results of all 5 experiments are summarized in Table I, which shows the total number of cultures falling in each arbitrary density class for each of the cell types studied.

As may be seen from this table, no statistically significant difference between the control and biotin-treated cultures was revealed

* This work, carried out under the direction of Dr. Paul Weiss, was done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and the University of Chicago, and has been aided by grants from the Dr. Wallace C. and Clara A. Abbott Memorial Fund of the University of Chicago.

1 Hamilton, H. L., and Plotz, H., PROC. SOC. EXP. BIOL. AND MED., 1942, **50**, 133.

2 Weiss, P., *Anat. Rec.*, 1934, **58**, 299.

3 Burt, A. S., *J. Cell. and Comp. Physiol.*, 1943, **21**, 145.

TABLE I.
Effect of 1/3 γ Biotin/cc Medium on Growth of Spinal Ganglia in Plasma.

Density class	Nerve fibers			Spindle cells			Macrophages		
	Control	Biotin	X^2	Control	Biotin	X^2	Control	Biotin	X^2
I	1	3	1.00	6	9	0.27	4	8	1.34
II	20	17	0.43	20	28	1.34	25	23	0.08
III	20	22	0.10	25	16	2.44	16	15	0.13
IV	12	13	0.00	2	2	0.00	8	9	0.00
Total	53	55	1.53	53	55	4.05	53	55	1.55
P between	0.95—0.50			0.20—0.10			0.95—0.50		

by use of the Chi Square method of analysis. The radius of cell outgrowth from each ganglion was measured with an ocular micrometer; and the average outgrowth from the controls was found to be 650 μ as compared with 602 μ for the biotin-treated ganglia. However, because of the large standard deviations involved, this difference is not significant.

As a further check, 72 cultures of spinal ganglia were made in a liquid medium consisting of 5 parts Tyrode's solution plus 1 part chicken serum, 1/3 γ biotin per cc being added to half the cultures and 50% alcohol equivalent to the volume of the biotin concentrate to the controls. In this medium, biotin appeared to have a pronounced inhibitory effect on all classes of cells. Nearly half of the biotin cultures showed no trace of growth, while the control ganglia produced many axons and a moderate number of spindle cells and macrophages.

Brain, though less adequate for tests of this kind, was examined in 10 cultures of 6-day embryonic chicks to which biotin was added and in 10 controls to which no vitamin was added. A narrow ring of mobilized nerve cells, such as was ascribed by Hamilton and Plotz to amitotic division, was present around most of the explants in biotin, and around most of the control cultures as well. Such a neuroblastic fringe is well known to be a passive result of plasma retraction in liquefying brain cultures[4,2] and cannot be considered evidence of nerve cell proliferation.

In order to determine possible biotin effects on tissue other than nerve, 20 explants of 6-day embryonic chick heart were made, 10 in biotin-plasma and 10 controls in alcohol-plasma. After 3 days' incubation, the radius of the growth zone was 944 μ in the controls and only 774 μ in the biotin cultures. Because of the large standard deviation obtained, the difference was not significant.

Discussion. The presence of alcohol in all cultures, biotin as well as controls, has depressed growth in comparison to several hundred alcohol-free cultures made in this laboratory for other purposes. This inhibitory effect of about 0.2% alcohol *in vitro* contrasts with findings in living tadpoles where the threshold for axon injury is over 0.5% and sheath cells are frequently unaffected by as much as 3% alcohol.[5] Furthermore, our cultures showed greater inhibition by the same concentration of alcohol in liquid medium than in plasma clots. Grossfeld[6] has reported a similar increase of sensitivity of heart fibroblasts to extreme pH values in liquid media as compared to plasma.

The presence of biotin in our cultures definitely had no stimulating effect on cell and fiber growth in plasma and depressed growth significantly in liquid medium. The discrepancy between these results and those of Hamilton and Plotz might be explained by possible variations in the chemicals used or by the different standards used in assessing growth. Their paper fails to give details of the criteria used in rating nerve fiber growth; moreover, in repeating their procedure in 16 paired cultures of complex tissue from the midline of 6-day-old chick embryos, I found that

[4] Olivo, O. M., *Arch. f. exp. Zellforsch.*, 1927, **4**, 43.

[5] Speidel, C. C., *J. Comp. Neur.*, 1936, **64**, 77.

[6] Grossfeld, H., *Z. f. Zellforsch. u. mikr. Anat.*, 1936, **25**, 312.

whatever axon growth did occur was almost completely obscured by other cells.

In the rat, neither biotin deficiency[7] nor administration of amounts of the vitamin far in excess of normal intake[8] has been found to affect the rate of regeneration of crushed sciatic nerves significantly. These findings in the living animal in conjunction with those reported here in tissue culture do not support the contention that biotin is a stimulant of axon growth.

Summary. In a total of 236 tissue cultures, no evidence was found for a growth stimulating action of biotin on embryonic chick axons, neuroblasts, spindle cells, or macrophages. In view of the close resemblance between axon regeneration *in vivo* and *in vitro*,[9] these results discourage the view that biotin might be clinically useful as a stimulant of nerve regeneration, at least during the crucial outgrowth phase.

7 Fischer, E., *Fed. Proc. Am. Soc. Exp. Biol.*, 1943, **2**, 13.

8 Lazere, B., Thomson, J. D., and Hines, H. M., PROC. SOC. EXP. BIOL. AND MED., 1943, **53**, 81.

9 Weiss, P., *Growth*, 1941, **5**, 163.

14362

Depression of Experimental Polycythemia by Stomach U.S.P.; Presence of Choline in Stomach U.S.P.*

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It was reported that the daily administration of 5 g of Stomach U.S.P. (Ventriculin†) to polycythemic animals had no effect on the red blood cell count, although the feeding of raw liver produced a reduction of erythrocytes.[1] Subsequently, we showed[2] that choline chloride depressed experimental polycythemia in the same manner as raw liver, and later[3] the probable mechanism of action was reported.

The present communication reports the results of the administration of a higher (10 g) daily dose of Ventriculin to polycythemic dogs, and the proof that Ventriculin contains choline which is probably the substance responsible for the depression of polycythemia.

Procedure. Two normal and 2 splenectomized dogs were maintained on a constant adequate diet. Polycythemia was produced in 2 of the animals by the daily oral administration of cobalt chloride according to a method described previously,[4] and the other 2 dogs were made polycythemic by the daily injection of posterior pituitary solution.[5] After the development of the polycythemias, each dog was fed 10 g of Ventriculin daily in addition to the erythropoietic-stimulating substances.

To determine the possible existence of choline in Ventriculin, a chemical analysis was made by the method of Johnson, Irvine, and Walton.[6] In addition, a pharmacological test was made by acetylating a deproteinized aqueous extract of Ventriculin according to the method of Mentzer *et al.*[7] and determining its effect on the blood pressure of a dog.

Results. Fig. 1 shows the effect of the daily oral administration of 10 g of Ventriculin upon the red blood cell counts of 4 polycythemic dogs. It will be noted that this

* Research paper No. 539, journal series, University of Arkansas.

† Supplied by Parke, Davis & Co., Detroit, Mich.

1 Davis, J. E., *Am. J. Physiol.*, 1938, **122**, 397.

2 Davis, J. E., *Am. J. Physiol.*, 1939, **127**, 322.

3 Davis, J. E., *J. Pharm. and Exp. Therap.*, 1940, **70**, 408.

4 Davis, J. E., PROC. SOC. EXP. BIOL. AND MED., 1937, **37**, 96.

5 Davis, J. E., *Am. J. Physiol.*, 1942, **137**, 699.

6 Johnson, C. G., Irvine, J. L., and Walton, C., *J. Biol. Chem.*, 1939, **131**, 425.

7 Mentzer, C., Corteggiani, E., and Carayon-Gentil, A., *Bull. soc. chim. biol.*, 1939, **21**, 503.

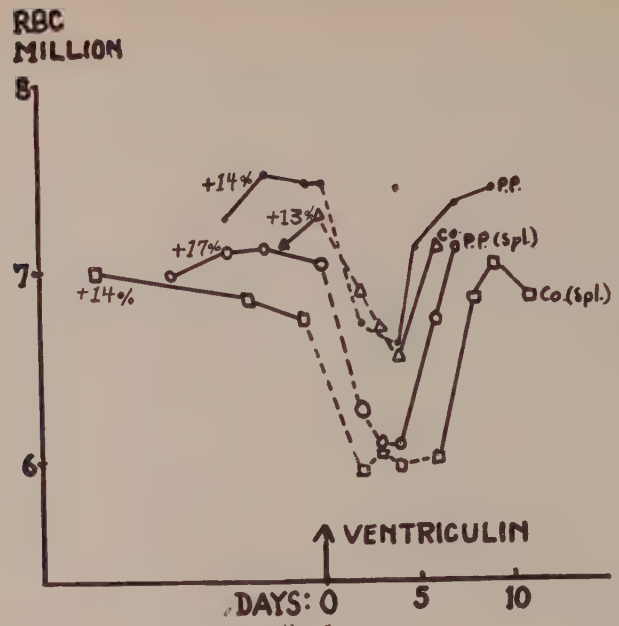

FIG. 1.

The Effect of the Daily Oral Administration of 10 Grams of Stomach, U.S.P., to 4 Polycythemic Dogs.

Figures at left of each line indicate degree of polycythemia in terms of percentage increase of red cell count above normal. Dashed lines show time during which Ventriculin was fed. P.P. = dog receiving posterior pituitary injections; C = cobalt-fed dog; Spl. = splenectomized animal.

procedure caused marked reductions in the erythrocyte numbers of all of the animals. Hemoglobin percentages (Hellige) were diminished proportionately, but leukocyte counts showed no uniform change and remained fairly constant. Upon cessation of stomach administration, the erythrocyte numbers and hemoglobin percentages returned to their polycythemic values.

The intravenous injection of acetylated extracts of ventriculin into anesthetized dogs caused precipitate decreases in blood pressure, amounting to more than 90 mm Hg. Lowering of the blood pressure did not occur when the acetylated extract was given after atropinization.

Discussion. The depression of polycythemia by stomach U.S.P. in these experiments appears to be similar to that produced by liver, choline,[2,3] and other vasodilator drugs.[8] Jacobs[9] has shown that a certain liver extract contains at least 1% of choline.

We believe that choline is the active constituent in liver that is responsible for the depression of erythropoiesis in polycythemia.[2] The possibility that choline is the common denominator in liver and stomach, as far as polycythemia depression is concerned, motivated us to search for choline in the antianemic stomach preparation. Our chemical extraction and determination yielded a choline reineckate product giving a value of about 1% choline in Ventriculin. Although this percentage of choline accounts very well for the effectiveness of Ventriculin in polycythemia, we doubt the purity of the reineckate compound obtained in the analysis. However, the vasodepressor action of acetylated extracts of Ventriculin, and its inhibition by atropine, would seem to prove conclusively that choline

[8] Davis, J. E., *J. Pharm. and Exp. Therap.*, 1941, **73**, 162.

[9] Jacobs, H. R., *J. Lab. and Clin. Med.*, 1938, **24**, 128.

is present in Ventriculin.

Conclusions. The daily oral administration of 10 g of Stomach U. S. P. to 4 polycythemic dogs caused depressions of their red blood cell counts. This preparation contains choline which is probably the constituent responsible for the depression of experimental polycythemia in these experiments.

The author wishes to thank Mr. Ralph Houghton for technical assistance rendered in the course of this work.

14363

Effect of Ephedrine Sulfate on the Red Blood Cell Count of Humans.*

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In 1941, the production of experimental polycythemia in dogs, rabbits, and man by the daily administration of ephedrine sulfate was reported by one of the authors.[1] Although the data on animals was adequate, it was possible to report data on only one human subject who received a daily dose of 50 mg of ephedrine sulfate. Erythropoiesis was apparently stimulated by the drug in this single individual, but the desire to have more data on more human subjects led us to make the investigation herein reported.

Procedure. Seven normal men who were leading a physically active life were used as subjects in this investigation. Red cell counts, leukocyte counts, and hemoglobin percentages (Sahli) were determined at frequent intervals before and during administration of the drug. After the normal blood cell values were obtained, each subject was given 50 mg of ephedrine sulfate daily by mouth. Fingertip blood samples were taken with the subject at *rest* and *before* the administration of the daily dose of the drug. Blood cell counts were made by an impartial technician who was unaware of the experimental procedures.

Results. Fig. 1 shows the effects of ephedrine sulfate administration for 3 to 4 weeks upon the red blood cell counts of all 7 subjects. It will be noted that the drug caused a rise of about one-half million (on the average) in the erythrocyte numbers. Hemoglobin percentages increased correspondingly, but leukocyte counts showed no uniform or constant change (not shown). In the subjects who could be observed for longer than 4 weeks, the erythrocyte counts fell back toward normal in spite of continued ephedrine administration.

Discussion. Although only one of the human subjects in these experiments showed a rise of 0.8 million (Fig. 1) which was comparable to the erythrocyte elevation from ephedrine in the subject reported previously[1] by Davis, it appears that ephedrine, in the daily dose of 50 mg produces a mild polycythemia. This polycythemia seems to be only temporary, a fact which we may perhaps attribute to the phenomenon of tachyphylaxie. It is also possible that these subjects, because of their physically active mode of life, showed weaker cardiovascular responses to ephedrine in the 50 mg dose. The mechanism of the ephedrine induced increase of erythropoiesis was postulated[1] as depending upon vasoconstriction and a curtailed blood and oxygen supply to bone barrow.

Conclusions. The daily oral administration of 50 mg of ephedrine sulfate to 7 normal men caused a mild but appreciable polycythemia within 3 weeks. The elevation of the red cell count appeared to be only temporary, since in some subjects it disappeared at some time after the fourth week of drug administration.

* Research paper No. 540, journal series, University of Arkansas.

[1] Davis, J. E., *Am. J. Physiol.*, 1941, **134**, 219.

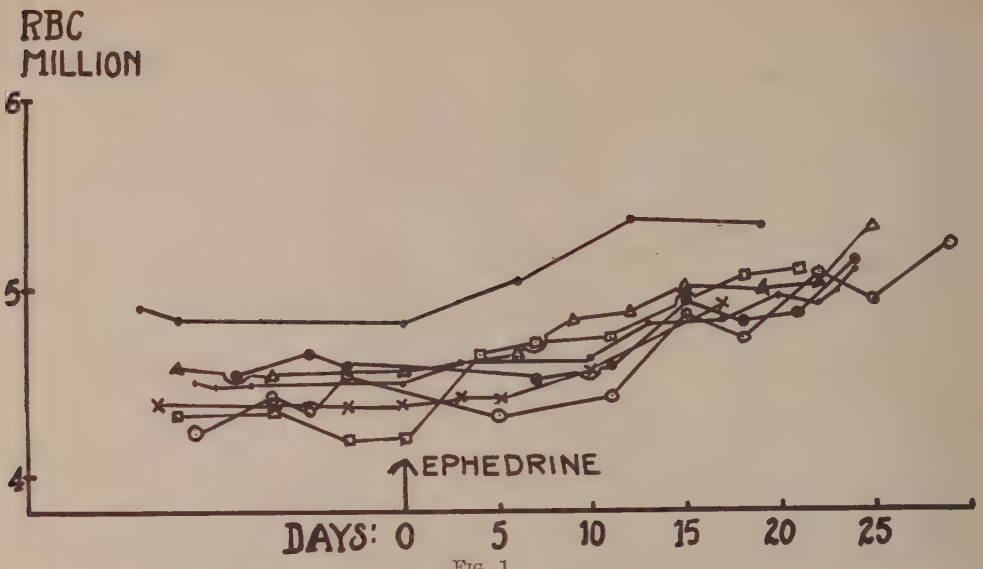

FIG. 1.
The effect of ephedrine sulfate (50 mg daily) upon the red blood cell counts of 7 normal men.

The continued daily oral administration of ephedrine sulfate (50 mg) produced no deleterious effects on the blood in these experiments.

14364

Deciduoma Formation with Desoxycorticosterone Acetate.*

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Until recently it was assumed that only progesterone or corpus luteum extracts are able to determine deciduoma formation in spayed animals primed with folliculoid compounds. This, if true, would be surprising especially in the case of ethynyl-testosterone and desoxycorticosterone acetate which, at relatively low dosages, elicit progestational changes in the uterus[1-4] and maintain pregnancy in spayed rabbits.[5,6] Contrary to the negative results obtained by Stolte[7] with ethynyl-testosterone, Cohen and Stein,[8] using the method proposed by Astwood,[9] succeeded in producing deciduomata in rats with a daily

* This work was made possible through a grant from the International Cancer Research Foundation. Desoxycorticosterone acetate was kindly supplied by Dr. Erwin Schwenk of the Schering Corporation of Bloomfield, N.J.

1 Inhoffen, H. H., Logemann, W., Hohlweg, W., and Serini, Arthur, *Ber. chem. Ges.*, 1938, **71**, 1024.

2 Emmens, C. W., and Parkes, A. S., *J. Endocrinol.*, 1939, **1**, 332.

3 Van Heuverswyn, J., Collins, V. J., Williams, W. L., and Gardner, W. U., PROC. SOC. EXP. BIOL. AND MED., 1939, **41**, 552.

4 Selye, H., and Masson, G., *J. Pharm. and Exp. Therap.*, 1943, **77**, 301.

5 Courrier, R., et Jost, A., *C. R. Soc. Biol.*, 1939, **130**, 1162.

6 Courrier, R., *Presse méd.*, 1940, **60-61**, 658.

7 Stolte, L. A. M., *Proc. nederl. Akad. Wetensch.*, 1940, **43**, 1242.

8 Cohen, M. R., and Stein, I. F., *Am. J. Obst. and Gynec.*, 1940, **40**, 713.

9 Astwood, E. B., *J. Endocrinol.*, 1939, **1**, 49.

oral dose of 10 mg of this steroid. Since no reports were made concerning desoxycorticosterone acetate, we thought that it would be of interest to know whether it is also able to produce artificial deciduoma.

Eighteen albino female rats weighing 150-200 g were divided into one experimental and one control group, each consisting of 9 animals. The procedure used was reported by Rothschild and Meyer[10] as giving a high percentage of positive results. The animals were spayed while in estrus, then treated with desoxycorticosterone acetate in a daily dose of 10 mg this being given in 2 subcutaneous injections of 0.2 cc of peanut oil. On the fifth day of treatment, the endometrium was traumatized with a suture needle. The hormone treatment was continued for 5 more days after which the animals were killed. The same procedure was used in the case of the animals of the control group except that cholesterol was given instead of desoxycorticosterone acetate.

At autopsy, all the experimental rats showed deciduomata (++++ reaction according to Astwood's scale except a ++ reaction in one sick animal). All the controls were negative.

Conclusions. In rats, desoxycorticosterone acetate is able to determine deciduoma formation in all 9 of the animals used, with a daily dose of 10 mg.

[10] Rothschild, I., and Meyer, R. K., *Physiol. Zool.* 1942, **15**, 216.

14365

A Tracer Study with Mn^{56} on Chicks with Perosis Produced by a Synthetic Manganese Deficient Diet.*

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A high calcium and phosphorus content in the diet of the chick results in the bone condition known as perosis.[1,2] The work of a number of investigators makes it apparent that under the above conditions, the disease is caused by an interference with the absorption of the manganese in the diet.[3,4] Administration of extra manganese, either orally or by injection, prevents the onset of perosis in the chicks reared on the high calcium and phosphorus diets.[3-6]

So far perosis has been produced only in birds reared on natural foodstuffs. It was of interest, therefore, to determine if the condition could be produced by a synthetic diet deficient in manganese, but of normal calcium and phosphorus content. In the experiments reported here, chicks were fed such a diet. The synthetic diet caused the development of the characteristic signs of perosis. The condition in the affected chicks did not differ in any particular form from the perosis caused by natural foodstuffs. It follows from this that primarily the deficiency of manganese and not any particular relationship between the manganese and calcium and phosphorus intake is responsible for the onset of perosis.

Tracer studies with Mn^{56} of the metabolism of manganese have yielded important information about the fate of this element in the body

* The synthetic chick ration used in this study was devised by Dr. H. J. Almquist of the Division of Poultry Husbandry of the University. We gratefully acknowledge our indebtedness to him for supplying us with the ration and for rearing the chicks.

[1] Titus, H. W., *Poul. Sci.*, 1932, **11**, 117.

[2] Hunter, J. E., Dutcher, R. A., and Knandel, H. C., *Poul. Sci.*, 1932, **11**, 239.

[3] Schaible, P. J., and Bandemer, S. A., *Poul. Sci.*, 1942, **21**, 8.

[4] Wilgus, H. S., Jr., Norris, L. C., and Heuser, G. F., *Science*, 1936, **84**, 252; *J. Nutr.*, 1937, **14**, 155.

[5] Lyons, M., Insko, W. M., Jr., and Martin, J. H., *Poul. Sci.*, 1938, **17**, 12, 264.

[6] Wilgus, H. S., Jr., and Patton, A. R., *J. Nutr.*, 1939, **18**, 35.

TABLE I.
Synthetic Chick Ration.*
Constituents in g per 100 g feed.

Basal Components		Salts	
Casein†	20	Calcium gluconate	8.0
Gelatin	5	$Ca_3(PO_4)_2$ precipitated	2.0
Wesson Oil	2.5	K_2HPO_4	0.5
Cod liver oil (U.S.P.)	1.0	KCl	0.3
Cellulose (Cellu-flour)	5.0	NaCl	1.0
Yeast Extract‡ equivalent to	5.0	$MgSO_4 \cdot 7H_2O$	0.3
Glucose (cerelose)	to 100	$Na_2SiO_3 \cdot 6H_2O$	0.2
Vitamin Supplements		$FeSO_4$	0.04
Cholic acid	0.1	$CuSO_4$	0.005
α-tocopherol	0.005	$ZnSO_4$	0.005
Thiaminchloride	0.001	$(Al)_2(SO_4)_3$	0.05
Riboflavin	0.001	KI	0.01
Pyridoxine	0.001	$Co(OOC\,CH_3)_2 \cdot 4H_2O$	0.002
Nicotinic acid	0.003		
Calcium pantothenate	0.004		
Biotin concentrate§	0.5		
Choline chloride	0.2		
Vitamin K‖			
Hydroquinone	0.001		

† Reprecipitated and dried with ethanol.
‡ 50% methanol extract concentrated to 1 ml = 4 g.
§ Biotin concentrate from S. M. A. Corp.
‖ 2 methyl-1,4-naphthohydroquinone-diphosphoric ester (tetra sodium salt).

of the mammal.[7-8] Because of the many differences in physiological characteristics between the mammal and the bird, a tracer study was conducted with Mn^{56} of the fate of manganese in the manganese-low chicks and their controls.

Experimental. Perosis. Newly hatched chicks were placed on the synthetic ration which had the composition shown in Table I. An analysis of this diet showed it contained 2.8 mg Mn per kilo of feed. The content of the control diet was 59.6 mg Mn per kilo.

The chicks placed on the manganese-low diet were retarded in weight and began to show evidence of perosis in about two weeks time. The leg bones of the deficient chicks were shorter in comparison to the width than the bones of the controls. A comparison of the weight and bone measurements are given in Table II. In several of the chicks the deformity due to the perosis progressed to such a degree that they were no longer able to stand up and feed.

Tracer Experiments. After the 2-week growth period, groups of 4 each of the deficient and control chicks were given the radioactive manganese. Each chick was given 1 ml of a solution containing 15.8 μg of Mn*† dissolved in 0.9% NaCl solution. The radioactive count in each ml of solution was 9200 per minute for Group I and 10,000 for Group II. Half of each group of chicks were given the Mn* orally, the other half by subcutaneous injection.‡ Each chick was placed in a separate metabolism cage and the excreta collected. They were sacrificed 72 hours after the administration of the radioactive material and the liver, kidney, and carcass as well as the excreta were analyzed for the distribution of the Mn*. The gastrointestinal tract was dissected out and added to the excreta of each chick. The results are given in Table III. The radioactivity of the samples was measured as described in previous publications.[7,8] Total manganese was determined by the colorimetric method of Wiese and Johnson.[9]

Discussion. The total radioactive man-

7 Greenberg, D. M., and Campbell, W. W., *Proc. Nat. Acad. Sci.*, 1940, **26**, 448.

8 Greenberg, D. M., Copp, D. H., and Cuthbertson, E. M., *J. Biol. Chem.*, 1943, **147**, 749.

† The asterisk is used to designate a labeled element.

‡ One animal of Group I died when injected intraperitoneally with the manganese-containing solution.

9 Wiese, A. C., and Johnson, B. C., *J. Biol. Chem.*, 1939, **127**, 203.

TABLE II.
Bone Measurements of Manganese-Deficient and Control Chicks.

	Tibia		Metatarsus		Weight of 2 tibia + 2 metatarsals (g)
	Length (cm)	Joint width (cm)	Length (cm)	Joint width (cm)	
			Deficient.		
Group I	3.77 ± 0.15 (6)	0.575 ± 0.03 (6)	2.77 ± 0.17 (6)	0.62 ± 0.03 (6)	0.81 ± 0.15 (3)
Group II	4.0 ± 0.12 (8)	0.77 ± 0.6 (8)	3.0 ± 0.07 (8)	0.73 ± 0.04 (8)	1.325 ± 0.30 (3)
			Controls.		
Group I	4.5 ± 0.20 (8)	0.60 ± 0.04 (8)	3.5 ± 0.15 (8)	0.60 ± 0.04 (8)	1.165 ± 0.24 (4)
Group II	4.9 ± 0.10 (8)	0.65 ± 0.04 (8)	3.45 ± 0.13 (8)	0.70 ± 0.03 (8)	1.505 ± 0.9 (3)

Measure of variability is mean deviation of the mean. Numbers in parentheses represent the number of samples.

TABLE III.
Partition of Labeled and Total Manganese in Manganese-Deficient and Control Chicks.*

	Excreta	Liver	Kidney	Carcass
		Labeled manganese in per cent of total dose.		
Deficient, Oral	96.3 ± 0.85 (4)	1.0 ± 0.14 (4)	0 (3)	2.4 ± 0.7 (4)
" Injected	65.0 ± 0.6 (3)	16.0 ± 0.33 (3)	2.3 ± 1.3 (3)	10.3 ± 0.7 (3)
Control, Oral	99.0 ± 0.05 (4)	0.3 ± 0.3 (4)	0 (3)	0.7 ± 0.17 (4)
" Injected	75.6 ± 3.1 (4)	3.5 ± 0.36 (4)	2.35 ± 0.2 (4)	18.2 ± 1.4 (4)
		Total manganese (mg per 100 g fresh tissue).		
Deficient		0.053 ± 0.012 (7)	0.05 ± 0.06 (6)	0.023 ± 0.03 (7)
Control		0.20 ± 0.026 (8)	0.22 ± 0.006 (7)	0.07 ± 0.015 (8)

* The figures represent mean values ± mean deviation. Numbers in parentheses are the number of chicks analyzed.

ganese recovered from each animal was computed from the measured activities of the excreta, carcass and the tissues analyzed separately. The recovery varied between 90 and 101% of the total radio-counts. To give a uniform basis of comparison, the data have been corrected in each case to a total value of 100.

Examination of the data of Table III shows that the major part of the administered dose of Mn*, little as it was, was excreted by both the manganese-low and the control chicks. This was true of the injected as well as the orally administered manganese. In the case of both routes of administration, however, the deficient chicks retained a greater proportion of the Mn*, than did the controls. Nearly all the tissues of the manganese-deficient birds showed a considerable retention of the administered Mn*, liver being the most conspicuous. Bone showed little participation in the turnover of the administered manganese. Measurable amounts of Mn* appeared only in the bone of the injected deficient chicks. No Mn* was demonstrable in the bone of either the manganese-low or control birds which were given the dose orally. The manganese requirement of bone must be extremely minute.

The greater accumulation of Mn* in the livers of the deficient over that of the control chicks signifies that manganese has an important role in liver function. It has been surmised that in the mammalian liver, manganese is required for the activation of the enzyme arginase. The liver of the chick does not contain any arginase. Evidently manganese is required by the liver for other processes besides arginase activation.

There was no differentiation in the uptake of Mn* by the kidney between the deficient and control chicks. The Mn* that appeared in the kidney presumably was intended for excretion since no Mn* was found in this organ when the element was administered orally.

Summary. Perosis has been produced in chicks reared on a synthetic manganese-deficient diet. Tracer experiments with labeled manganese show that the major part of the manganese, whether administered orally or by injection, is excreted by both the deficient and control chicks. The liver is the

most active participant in the body in the metabolism of manganese. The uptake of labeled manganese by bone was not measurable when the dose was administered orally.

14366

Relaxation of the Pelvic Ligaments of Castrate Hysterectomized Guinea Pigs Induced by Progesterone.*

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Haterius and Fugo[1] showed that in castrate female guinea pigs relaxation of the pelvic ligaments could be elicited by treatment with crystalline progesterone after preliminary conditioning with estrogens. These findings cast some doubt upon the existence of a separate relaxation hormone, or at least its indispensability in the process of pelvic relaxation.

More recently Hisaw *et al.*[2] confirmed the ability of crystalline progesterone to relax the pelvic ligaments in the spayed and estrogenized guinea pig. But they reported that in castrates which were hysterectomized the estrogen-progesterone treatment did not induce relaxation. The authors concluded therefore that under the conditions of their experiments "the uterus is indispensable for the formation of relaxin."

It would seem from this interesting report that the presence of the uterus in some unexplained way facilitates relaxation but is not a necessary factor in the development of this reaction. Similarly progesterone is not indispensable since relaxin alone produces relaxation in the estrogenized hysterectomized castrate. Surprisingly, however, the combination of two factors (uterus and progesterone) each shown to be nonessential, together give a positive reaction.

It was not the intention of this study to analyze the complicated nature of this reaction. Rather it was thought necessary to reinvestigate our earlier conclusion, namely, that progesterone alone was sufficient to produce relaxation when given in combination with estrogen.

Furthermore it occurred to us that perhaps the failure of Hisaw *et al.* to obtain relaxation in castrate hysterectomized guinea pigs might

TABLE I.
Summary of Cases of Relaxation Following Estrogen-progesterone Treatment in Castrated-hysterectomized Guinea Pigs.

Guinea pig No.	1	2	3	4	5	6	7	8	9*	10*	11*	12*
0.5 mg Est. Diprop. daily (days)	7	7	7	7	7	7	7	7	0	0	0	0
0.5 mg Est. Diprop. + 0.5 mg Prog. daily (days)	7	7	7	7	7	7	7	7	0	0	0	0
1.0 mg Est. Diprop. + 1.0 mg Prog. daily (days)	4	2	2	3	0	1	3	0	0	0	0	0
Total mg Est. Diprop.	11.0	9.0	9.0	10.0	7.0	8.0	10.0	7.0	0	0	0	0
Total mg Prog.	7.5	5.5	5.5	6.5	3.5	4.5	6.5	3.5	0	0	0	0
Day Relax. occurred after init. inject.	19	17	17	18	15	16	18	15	N.R.	N.R.	N.R.	N.R.

N.R. = No relaxation.
*Castrated and hysterectomized.

* Aided by grants from the National Research Council, Committee for Research in Problems of Sex; grant administered by Professor Emil Witschi.

[1] Haterius, H. O., and Fugo, N. W., PROC. SOC. EXP. BIOL. AND MED., 1939, **42**, 155.

[2] Hisaw, F. L., Zarrow, M. X., Talmage, R. V. N., Money, W. L., and Abramowitz, A. A., *Anat. Rec.*, 1942, **84**, 457.

be due to a time factor since their experimental procedure consisted in the injection of 250 mg of estradiol on 4 consecutive days followed by a single injection of 5 to 10 mg of progesterone on the following day.

Consequently 12 virgin guinea pigs weighing approximately 200 g were castrated and hysterectomized under ether anesthesia. A period of 17 days was allowed to elapse between the operation and the initiation of the hormonal injections to assure the complete recovery of the animal and the recession of all sexual characteristics. At this time subcutaneous injections of estrogen were made for 7 days followed by a period of estrogen-progesterone treatment. Dosages and details of administration are listed in Table I. Relaxation of the pelvic ligaments occurred in all injected animals between the 15th and 19th day of hormone administration.

The last 4 experimental animals were castrated and hysterectomized but received no hormone treatment. None of these animals showed any relaxation during the post-operative period of 37 days.

It appears from this experiment that castrated and hysterectomized virgin guinea pigs when given progesterone after an initial period of estrogen treatment show relaxation of the pelvic ligaments in the complete absence of both ovaries and uteri.

14367

Natural Resistance and Susceptibility to Russian Spring-Summer Encephalitis in Mice.*

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Webster[1] demonstrated that resistance and susceptibility in mice to *B. enteritidis* are inherited independently of resistance and susceptibility to St. Louis encephalitis virus in the same species. Consequently, by inbreeding and selection from a hybrid stock, he was able to derive 4 strains of mice exhibiting the 4 possible combinations of resistance and susceptibility to these two pathogens. For convenience these stable, uniform, inbred strains were designated as follows: Bacteria-resistant-virus-susceptible (BRVS) and, in the same manner, the three remaining strains, BRVR, BSVS, BSVR. These designations were valid as well in terms of the louping-ill virus in the instance of the BSVR and BRVS lines,[2] but all strains were uniformly susceptible to mouse passage rabies virus,[1] to vesicular stomatitis virus,[3] and to lymphocytic choriomeningitis virus.[4] At the present time, then, designation of these strains of mice as "virus-resistant" or "virus-susceptible" is restricted to the viruses of St. Louis encephalitis and louping-ill.

Recently, Casals and Webster[5] have demonstrated the existence of a close relationship between the viruses of louping-ill and Russian spring-summer encephalitis, a relationship based on similarities in (1) pathogenicity for various hosts, (2) complement-fixing antibody formation, (3) neutralizing antibody formation, and (4) cross-resistance following vaccination. The question arose as to whether this relationship could be further defined to include (5) natural resistance. This was

* These investigations were aided through the Commission on Neurotropic Virus Diseases, Board for the Investigation and Control of Influenza and Other Epidemic Diseases in the Army, Preventive Medicine Division, Office of the Surgeon General, United States Army.

1 Webster, L. T., *J. Exp. Med.*, 1937, **65**, 261.

2 Webster, L. T., *J. Exp. Med.*, 1933, **57**, 819.

3 Olitsky, Peter K., Sabin, Albert B., and Cox, Herald R., *J. Exp. Med.*, 1936, **64**, 723.

4 Casals, J., unpublished data.

5 Casals, J., and Webster, L. T., *Science*, 1943, **97**, 246.

TABLE I.
Inoculation of Russian Spring-Summer Encephalitis Virus in Different Strains of Mice.

Strain of mice	Fate of mice following subcutaneous injection of Russian spring-summer encephalitis virus in dilution:						
	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	10^{-10}
BRVR	S,S,S,S	S,S,S,S	S,S,S,S	S,S,S,S	S,S,S,S	S,S,S,S	
BSVR	S,S,S,S	S,S,S,S	S,S,S,S	S,S,S,S	S,S,S,S	S,S,S,S	
BSVS	8,8,8,9	9,9,9,10	9,9,15,S	10,10,12,S	9,10,12,S	S,S,S,S	S,S,S,S
BRVS	8,8,8,8	7,8,8,9	7,8,8,9	8,8,9,9	8,9,11,12	S,S,S,S	S,S,S,S
W-Swiss		9,9,11,11	8,9,10,13	8,9,13,S	9,12,12,S	15,S,S,S	12,20,S,S

TABLE II.
LD_{50} and Resistance Indices of Mice Following Injection of Russian Spring-Summer Encephalitis Virus by Subcutaneous or Intracerebral Routes.

Strain of mice	LD_{50}% (intracerebral)	Resistance index (intracerebral)	LD_{50}% (subcutaneous)	Resistance index (subcutaneous)
BRVR	$<10^{-3}$	>2,000,000	$<10^{-2}$	>10,000,000
BSVR			$<10^{-4}$	>100,000
BSVS			10^{-8}	10
BRVS	$10^{-9.8}$	—3	$10^{-8.3}$	5
W-Swiss	$10^{-9.3}$	1	10^{-9}	1

readily determined by testing the resistance and susceptibility of the 4 Webster strains mentioned above.

A number of tests were carried out in the following manner: Serial tenfold dilutions of mouse brain infected with Russian spring-summer encephalitis virus were made up in normal rabbit serum in the usual way, using physiological saline solution as diluent; then batches of mice from different strains were tested subcutaneously or intracerebrally by injection of 0.5 cc and 0.03 cc respectively of the several dilutions of virus. Unselected W-Swiss mice were included in each test as known susceptible controls. Four mice per dilution were infected and all animals employed in the tests were of the same age. Following injection the mice were kept under observation for 28 days and the survivors discarded.

Table I shows the results of one of the experiments. The figures in each column indicate the day of death of the mice; "S" represents those that survived the infection.

Other tests carried out under comparable conditions gave similar results which are summarized in Table II. This table gives the LD_{50} (50% mortality endpoint dilution) obtained by using the Reed and Muench method,[6] as well as the Resistance Index (maximum number of fatal doses resisted) with relation to the control W-Swiss mice, whose Resistance Index is 1.

Since Resistance Indices of 10 or less are not significant, the values obtained in the tests indicated that BSVS and BRVS mice were susceptible to inoculation of Russian spring-summer encephalitis virus to the same extent as W-Swiss unselected mice, while BSVR and BRVR mice were resistant to inoculation of more than 2,000,000 intracerebral doses and to more than 10,000,000 subcutaneous doses of virus lethal to either W-Swiss or BSVS and BRVS strains.

Conclusion. Strains of mice selectively bred for susceptibility and resistance to louping-ill and St. Louis encephalitis viruses proved to be similarly susceptible and resistant to subcutaneous or intracerebral inoculation of Russian spring-summer encephalitis virus.

[6] Reed, L. J., and Muench, H., *Am. J. Hyg.*, 1938, **27**, 493.

14368 P

Effect of Yeast Extracts on Local Anesthetic Activity of Cocaine.

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In connection with studies of the effects of yeast and tissue extracts on the metabolism of microörganisms and tissues we have observed that yeast extracts having the property of increasing respiration offset the toxicity of germicides for molds,[1] rat skin,[2] and yeast.[3] Yeast and tissue extracts also exert antisulfanilamide effects, presumably largely due to the content of *p*-aminobenzoic acid[4] and other substances.[5] Exploratory experiments are being conducted on the possible antagonism of yeast extracts for other physiologically active compounds and the present note reports preliminary work on such antagonism for cocaine.

For this work an aqueous-alcoholic extract of Fleischmann's bakers' yeast was prepared as described previously[6] and corresponded to Fraction A of the earlier work.[6] The local anesthetic activity of 0.05 M (1.7%) cocaine hydrochloride with or without yeast extract was determined on the rabbit cornea after 1-minute instillation of the solution according to the usual technic.[7] Four rabbits were used after first standardizing them with cocaine. At least a week elapsed between successive experiments on the same rabbit to allow complete recovery and prevent any tolerance effects. The experiments are summarized in Table I.

Discussion. The 30-minute duration of anesthesia of the rabbit cornea caused by a 1-minute instillation of 0.05 M cocaine hydrochloride compares satisfactorily with other values reported in the literature.[7,8] Addition of 1% of yeast extract caused a lowering of average duration to 27 minutes, and increasing the concentration of yeast extract to 5% further lowered the average anesthetic duration to 24 minutes.

A reason for the relatively small decrease of activity becomes apparent when it is observed that the addition of the yeast extract raised the pH of the cocaine hydrochloride from 4.8 to 6.1 or 6.2. It is well known that alkalinization of cocaine salt solutions increases their anesthetic activity, presumably due to the release of the free base. Régnier has published data on the effect of pH on the local anesthetic activity of cocaine hydrochloride[9] using a rabbit cornea method which differs from ours in technic[10] but which can be used satisfactorily for comparison. Computations based on Régnier's data indicate that an increase in the pH of cocaine solutions from 4.8 to 6.2 should increase the duration of anesthesia by 24%. This would give a theoretical duration of 37 minutes for 0.05 M cocaine hydrochloride at a pH of 6.2. Since 0.05 M cocaine hydrochloride plus 5% of yeast extract at a pH of 6.2 gave a duration of 24 minutes, this decrease would amount to

* The assistance of Daniel Whalen in some of the experiments is gratefully acknowledged.

1 Cook, E. S., and Kreke, C. W., *Nature*, 1940, **146**, 688.

2 Cook, E. S., Kreke, C. W., Eilert, M. R., and Sawyer, M. A., PROC. SOC. EXP. BIOL. AND MED., 1942, **50**, 210.

3 Cook, E. S., and Kreke, C. W., PROC. SOC. EXP. BIOL. AND MED., 1943, **53**, 222.

4 Dubos, R., *Ann. Rev. Biochem.*, 1942, **11**, 662.

5 Harris, J. S., and Kohn, H. I., *J. Pharm. and Exp. Therap.*, 1941, **73**, 383; Martin, G. J., and Fisher, C. V., *J. Biol. Chem.*, 1942, **144**, 289; Snell, E. E., and Mitchell, H. K., *Arch. Biochem.*, 1942, **1**, 93.

6 Cook, E. S., Kreke, C. W., and Nutini, L. G., *Studies Inst. Divi Thomae*, 1938, **2**, 23.

7 Rider, T. H., *J. Pharm. and Exp. Therap.*, 1930, **39**, 329; Rider, T. H., with Cook, E. S., *Ibid.*, 1938, **64**, 1.

8 Schmitz, H. L., and Loevenhart, A. S., *J. Pharm. and Exp. Therap.*, 1924, **24**, 162.

9 Régnier, J., *Compt. rend.*, 1924, **179**, 354; *Bull. sci. Pharmacol.*, 1924, **31**, 513.

10 Régnier, J., *Méthodes de Mesure de l'Activité des Anesthésiques Locaux*, Andre Brulliard, Saint Dizier, 1929.

TABLE I.
Effects of Yeast Extract on Cocaine Anesthesia of Rabbit Cornea (0.05 M Cocaine · HCl).

Yeast extr. conc., %	pH	Avg duration of anesthesia, min.	No. of detns.	% decrease in duration of anesthesia
0	4.8	30.0	16	
1	6.1	27.0	8	10
5	6.2	24.0	8	20
5	4.8	13.5	14	55

35% of the theoretical value. When the 0.05 M cocaine hydrochloride solution containing 5% of yeast extract was adjusted to a pH of 4.8 with hydrochloric acid, an average anesthetic duration of 13.5 minutes was obtained, a decrease over the control of 55%. It is evident that the yeast extract lowers the corneal anesthetic activity of cocaine and that this lowering becomes striking when compensation is made for pH changes.

Experiments, too few for a detailed report, indicated that the yeast extract lowered the subcutaneous LD_{50} of cocaine for rats by about 20% when cocaine and yeast extract were injected simultaneously in equal dosage on opposite sides of the abdomen. Further work is being undertaken on local anesthetics and on other physiologically active compounds.

Summary. Addition of a yeast extract to cocaine hydrochloride solution reduces the corneal anesthetic potency, this reduction being especially notable when the pH of the treated solution is lowered to the level of the control solution. There is evidence that the subcutaneous toxicity of cocaine is also decreased by the extract.

14369

Angiotonin Myotropism

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Page and Helmer[1] stated that angiotonin exhibited smooth-muscle stimulating properties since it constricted blood vessels of the perfused rabbit ear, caused contraction of isolated segments of rabbit ileum and elevated arterial tension in the pithed cat. Apparently no effort was made in their vascular experiments to determine if angiotonin acted myotropically or by way of sympathetic vasoconstrictor components. Another approach to this problem is afforded by the technic employed in our experiments involving sympatholysis.*

Method. Ten cats and 2 dogs were anesthetized either with urethane gastrically or pentobarbital intravenously and then prepared for the insertion of cannulæ into the trachea, femoral vein and carotid artery. In 4 experiments Wharton's duct was also cannulated to determine the effect of angiotonin upon submaxillary salivation. Salivary flow was recorded manometrically. Blood pressure was recorded from the carotid artery with a mercury manometer (Gorrell's plastic tube type) and all drugs were infused intravenously in physiologic saline solution.

Drugs were administered in the following order and usually in the following dosage in mg or cc per kg: Epinephrine HCl, 0.015 mg;

[1] Page, I. H., and Helmer, O. M., *J. Exp. Med.*, 1939, **71**, 29.

* We wish to acknowledge the cooperation in this project of Dr. Donel Sullivan of Detroit Receiving Hospital and Dr. Robert Byberg of Henry Ford Hospital, both former assistants in Pharmacology.

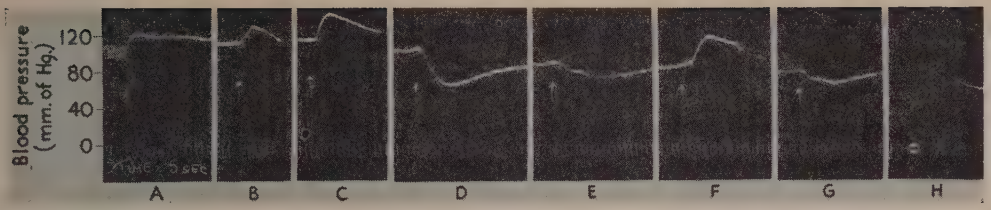

FIG. 1.

Cat, 2.7 kg, 12-16-42. Blood pressure reactions under urethane anesthesia. Time—5 seconds. All doses on per kilogram basis.

A. Section of vagi; B. epinephrine, 0.015 mg; C. angiotonin, 0.05 cc; D. ethyl yohimbine, 3 mg; E. epinephrine, 0.015 mg; F. angiotonin, 0.05 cc; G. epinephrine, 0.015 mg; H. pituitrin, 0.07 cc.

Angiotonin,† 0.05 cc; Ethyl Yohimbine HCl,‡ 3 mg; Angiotonin, 0.05 cc; Epinephrine HCl, 0.015 mg; Pituitrin, 0.07 cc.

Results. The typical reactions to the drugs employed differed quantitatively but were qualitatively uniform. Epinephrine and angiotonin caused the anticipated rises in arterial tension when injected prior to ethyl yohimbine but after the latter drug epinephrine produced its customary fall as has been previously reported.[2,3] Angiotonin, on the other hand, persistently effected a rise in arterial tension not unlike that produced before ethyl yohimbine and similar to that of pituitrin.

Angiotonin had no effect upon salivation and produced no pupillary reactions.

Discussion and Comment. Ethyl Yohimbine is an adrenolytic and sympatholytic§ agent and hence acts as a "medical scalpel" in relation to sympathetic vasoconstrictor neural components of the vascular system. This nullification of sympathetic vasoconstrictors extends likewise to the hypertensive state.[4] Sympathetic inhibition is evident from Fig. 1 in which the usual rise in arterial tension produced by epinephrine (B) is reversed by the yohimbine radicle (D). The sympathetic vasoconstrictor elements have been nullified by ethyl yohimbine, leaving the vasodilator mechanisms under the influence of epinephrine and resulting in a drop in arterial pressure (E). Angiotonin (F), however, still produces a rise in blood pressure which may be accounted for by either of two hypotheses: either (a) angiotonin acts directly upon smooth muscle components as do both soluble barium salts and pituitrin (H) or (b) angiotonin nullifies the vasoconstrictor paralyzing or sympatholytic action of ethyl yohimbine and acts adrenergically like epinephrine. The latter seems not to be the case since epinephrine (G), following ethyl yohimbine and angiotonin in the order named, still has its action "reversed." This indicates that a paralyzing action of ethyl yohimbine still persists *after* angiotonin has been injected and implies that this paralysis must have prevailed *during* its injection. In other words, sympatholysis prevailed during the second injection of angiotonin as well as during the second injection of epinephrine. Had it not, epinephrine should have produced its usual rise in tension as it did prior to ethyl yohimbine.

Since pituitrin and angiotonin both produce a rise in arterial tension during the adreno-

† Angiotonin was supplied to us by courtesy of Dr. Irvine H. Page of the Lilly Laboratory for Clinical Research, Indianapolis, Indiana, in solutions of such strength that 0.2 cc produced a rise of approximately 30 mm Hg. in the anesthetized dog weighing about 10 kilograms.

‡ Ethyl Yohimbine HCl was an 8-year-old sample but active and was originally supplied to us by Hoffmann-LaRoche Company through courtesy of Drs. A. G. Young and D. Worrall of Boston, Mass.

[2] Hamet, *C. R. Acad. d sc.*, 1925, **180**, 2074.

[3] Young, A. G., and Yonkman, F. F., *J. Pharm. and Exp. Therap.*, 1936, **57**, 150.

§ There has been some discussion regarding sympatholysis by Yohimbine and its congners and evidence supporting a true sympatholytic, as well as adrenolytic action, will be presented in another communication.

[4] Chase, H. F., Yonkman, F. F., and Lehman, A. J., *J. Pharm. and Exp. Therap.*, 1941, **72**, 6.

lytic or sympatholytic action of ethyl yohimbine it seems reasonable to believe that they act directly upon smooth muscle components and not upon ephinephrine-receptors in the vascular musculature.

The belief that epinephrine-receptors are not stimulated by angiotonin gains support by the failure of this substance to produce salivary flow or mydriasis in those cats which salivated profusely after epinephrine and cervical sympathetic nerve faradization.

The results of our vascular experiments confirm those reported by other workers[5] in that this hypertensive agent is unlike epinephrine. In their investigations a dioxane derivative, F 933, was employed as the sympatholytic agent and their angiotonin equivalent was known as pepsitensin which seems to be similar to the "hypertensin" of Braun Menendez and his co-workers.[6]

Conclusions. 1. Angiotonin (Page) has a true pituitrin-like action. 2. It contracts smooth muscle directly and not through the medium of adrenergic neural vasoconstrictor components. 3. Its action is not reversed by ethyl yohimbine as is that of epinephrine. 4. It exhibits no vasodilating action such as is characteristic of small doses of epinephrine. 5. It does not nullify the adrenolytic or sympatholytic action of ethyl yohimbine. 6. It has no effect upon cholinergically or adrenergically controlled salivation or pupillary responses.

5 Alanso, O., Croxatto, R., and Croxatto, H., PROC. SOC. EXP. BIOL. AND MED., 1943, **52**, 61.

6 Menendez, B., Fasciolo, J. C., Leloir, L. F., and Munoz, J. M., *J. Physiol.*, 1940, **98**, 283.

14370 P

Effect of Insulin, Glucose, and Glucose and Insulin on the Rate of Metabolism of Ethyl Alcohol.

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Reports in the literature are not in agreement concerning the effect of insulin on the rate of metabolism of ethyl alcohol. Numerous reports indicate that insulin has no effect on the rate of metabolism of this substance.[1–7] Many reports claim, on the other hand, that insulin or insulin and glucose increase the rate of oxidation of ethyl alcohol.[3,8–14]

The conflicting reports in the literature, the fact that insulin or insulin and glucose have been used clinically in the treatment of acute alcoholism, and the theoretical unlikelihood that insulin would be so lacking in specificity

1 Hirschfelder, A. D., and Maxwell, H. C., *Am. J. Physiol.*, 1924, **70**, 520.

2 Fleming, R., and Reynolds, D., *J. Pharm. and Exp. Therap.*, 1935, **54**, 236.

3 Goldfarb, W., Bowman, K. M., and Parker, S., *J. Clin. Invest.*, 1939, **18**, 581.

4 Bohmer, K., *Deutsche Z. f. d. ges. gerichtl. Med.*, 1938, **30**, 205.

5 Lang, S., and Von Schlick, B., *Z. Exp. Med.*, 1936, **99**, 81.

6 Dontcheff, L., *C. R. Soc. Biol.*, 1939, **130**, 1406.

7 Mirsky, I. A., and Nelson, N., *Am. J. Physiol.*, 1939, **127**, 308.

8 Supniewski, J. V., *J. Biol. Chem.*, 1926, **70**, 13.

9 Newman, H. W., and Cutting, W. C., *J. Clin. Invest.*, 1935, **14**, 945.

10 Widmark, E. M. P., *Biochem. Z.*, 1935, **282**, 79.

11 Bickel, A., *Deutsche Med. Wchnschr.*, 1936, **62**, 1209.

12 Siegmund, B., and Flohr, W., *Klin. Wchnschr.*, 1937, **16**, 1718.

13 Clark, B. B., and Morrissey, R. W., *Am. J. Physiol.*, 1938 (P), **123**, 37.

14 Clark, B. B., Morrissey, R. W., Fazekas, J. F., and Welch, C. S., *Quart. J. Studies on Alcohol*, 1941, **1**, 663.

of action have led us to reinvestigate this problem.

In general, much of the published work may be criticized on the basis of the short time over which observations were made. And the clinical evidence is not well controlled in view of the poor state of nutrition often seen in patients with alcoholic intoxication serious enough to require hospitalization. In addition, some of the published work failed to show a study of the capacity of the animal or intoxicated man to oxidize alcohol over a control period with alcohol alone from the beginning to disappearance of the alcohol from the blood. Furthermore, much of the experimental work and all of the clinical work was done following the ingestion of alcohol by mouth. The variability of absorption rate in any animal and in different animals could cause apparent differences in the rate of disappearance of the alcohol from the blood. This would be true particularly when observations were made over only a few hours' time.

Methods. All studies were made on dogs which had been on a diet of Purina Dog Chow 2 or more days and then fasted 15 or more hours before the administration of alcohol in both the control and the experimental group which received insulin. These were carried out initially following administration of 3 cc

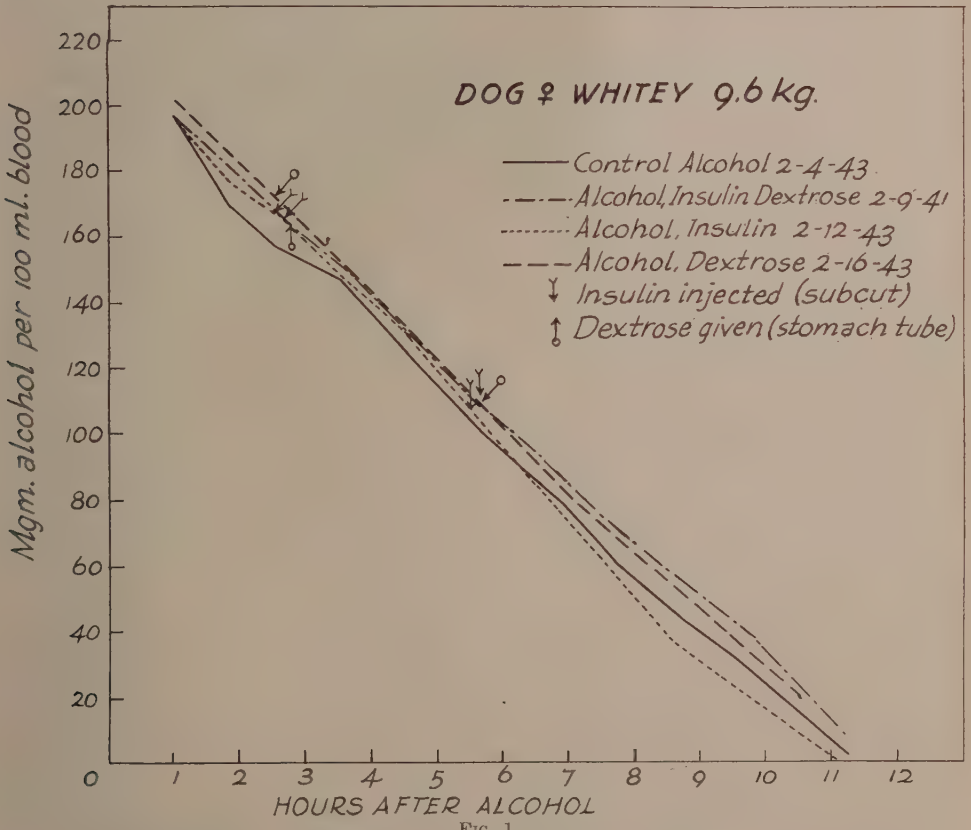

FIG. 1.

This shows the rate of disappearance of ethyl alcohol from the blood of a dog after intravenous administration of 20% ethyl alcohol, the total amount of which was 2 cc of absolute ethyl alcohol per kilo of weight, under the 4 conditions of alcohol alone, alcohol plus insulin, alcohol plus dextrose, alcohol plus insulin and dextrose. The dextrose was given in the amount of 3 g per kilo of weight as a 33⅓% solution. The dates upon which the 4 different experiments were done are indicated.

of ethyl alcohol per kilo by stomach tube. The alcohol was diluted to 20% strength by volume. The blood alcohol levels were so variable and the disappearance curves were so irregular that we concluded this to be an unsatisfactory method of giving the alcohol. We injected, therefore, ethyl alcohol intravenously in amounts of 2 cc per kilo in 20% strength. About 5 minutes were required for the injections. This method of administration gave disappearance curves that were smooth and reproducible in all animals in which it was repeated. About 11 hours were uniformly required for the complete disappearance of the alcohol from the blood.

Six dogs were subjected to the following experiments, usually in this sequence: (a) rate of disappearance of alcohol alone, (b) rate of disappearance of alcohol after the subcutaneous injection of one unit of regular insulin per kilogram. This dose was repeated after 3 hours, (c) rate of disappearance of alcohol after administration of 3 g of glucose per kilo by stomach tube as a 33⅓% solution, (d) rate of disappearance of alcohol plus insulin, as indicated in (b), plus glucose, as indicated in (c). In other words, 24 experiments were done on 6 dogs. In each instance 45 to 60 minutes were allowed to elapse following intravenous injection of alcohol to insure uniform distribution in the body. Blood specimens for alcohol analysis were then drawn at three-quarter- to one-hour intervals until the alcohol had practically disappeared. This was almost uniformly approximately 11 hours after injection of the alcohol. Estimations of alcohol in the blood were made according to a combination of the methods of Harger[15] and Heise.[16] The oxidation and titration were done by the former method and the distillation was accomplished by the latter method slightly modified. Combination of the two methods has been previously employed by Ewing.[17]

Results. The results and procedures of 4 experiments on one dog are shown graphically in Fig. 1. The results of the same types of experiments on 5 other dogs were practically identical. There is also evidence from some of our other experiments that the state of nutrition of the animal has some influence on the rate at which ethyl alcohol is metabolized. It is suggested on this basis that the general treatment of intoxicated patients with fluids and glucose, rather than the insulin, is responsible for the apparent increased rate of disappearance of ethyl alcohol from the blood of intoxicated man. It is further suggested that the correction of dehydration which is usually associated with severe intoxication may have a "diluting" effect on the alcohol of the body fluids and in this way give rise to an apparent increased rate of disappearance from the blood.

Conclusions. There is no evidence from 24 experiments in 6 dogs that insulin, glucose, or insulin plus glucose increases the rate of metabolism of ethyl alcohol.

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[15] Harger, R. N., *J. Lab. and Clin. Med.*, 1935, **20**, 746.

[16] Heise, H., *Am. J. Clin. Path.*, 1934, **4**, 182.

[17] Ewing, P. L., *Quart. J. Studies on Alcohol*, 1940, **1**, 483.

14371 P

Effect of Sodium Pyruvate on the Rate of Metabolism of Ethyl Alcohol.

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Westerfeld, Stotz and Berg[1,2] reported an average increase of 260% in the rate of ethyl alcohol metabolism following the oral administration of two 5 g doses of sodium pyruvate to dogs which had received 2 to 3 cc of absolute alcohol per kilogram by stomach tube 4 to 6 hours earlier. They stated also that feeding 5 g of sodium pyruvate to a dog which had not received alcohol previously resulted in an increase of venous blood pyruvate from 1.5 mg % to 4.5 mg %. When given to a dog that had received alcohol, pyruvate was utilized so rapidly, according to their claim, that it produced only a slight increase in blood pyruvate.

Since this reported effect on alcohol metabolism is so striking, it was decided to ascertain whether these results could be duplicated in dogs that received alcohol intravenously in the control experiment to establish the normal rate of metabolism of alcohol as well as for the experimental group which received sodium pyruvate.

We believe that the above reported experiments are open to criticism because of the short duration, the control and experimental observations were made during a single administration of alcohol, and the alcohol was given by mouth. Accordingly, we made control observations of the rate of alcohol metabolism over about 12 hours and experimental observations with pyruvate several days later over the same period of time. In all of our experiments, the alcohol was given intravenously to prevent any discrepancy due to a possible variable rate of absorption of alcohol from the gastrointestinal tract. Pyruvate was administered intravenously in some animals to insure its reaching the blood stream.

Methods. Twenty experiments were done on 6 dogs by determining the rate of disappearance from the blood of alcohol given intravenously in amounts of 2 cc of absolute alcohol per kilogram, diluted to 20%. All dogs were fed Purina Dog Chow for 2 or more days and fasted for at least 15 hours before each experiment. The animals were later given the same amount of alcohol intravenously and 2 doses of 0.5 g of sodium pyruvate per kilogram by stomach tube. The pyruvate was also given intravenously to 2 dogs. Blood samples for alcohol and pyruvate estimations were drawn at approximately hourly intervals, allowing 45 to 60 minutes after administration for uniform distribution. Specimens were taken at 15-minute intervals following pyruvate administration because of the rapid disappearance of the pyruvate. Pyruvate was estimated by the method of Bueding and Wortis[3] except that the blood was drawn and precipitated immediately according to the method of Friedemann and Haugen.[4] Alcohol estimations were made by the methods of Harger,[5] Heise,[6] and Ewing.[7]

Results. Fig. 1 shows graphically the rate of disappearance of ethyl alcohol alone and following 2 doses of 0.5 g per kilo of sodium pyruvate as a 5% solution by stomach tube. The pyruvate levels in the blood rose only slightly and transiently. It will be noted that administration of pyruvate had no influence on the rate of disappearance of ethyl alcohol from the blood.

1 Westerfeld, W. W., Stotz, E., and Berg, R., *Fed. Proc.*, 1942, **1**, 140.

2 Westerfeld, W. W., Stotz, E., and Berg, R., *J. Biol. Chem.*, 1942, **144**, 657.

3 Bueding, E., and Wortis, H., *J. Biol. Chem.*, 1940, **133**, 585.

4 Friedemann, T. E., and Haugen, G. E., *J. Biol. Chem.*, 1942, **144**, 67.

5 Harger, R. N., *J. Lab. and Clin. Med.*, 1935, **20**, 746.

6 Heise, H., *Am. J. Clin. Path.*, 1934, **4**, 182.

7 Ewing, P. L., *Quart. J. Studies on Alcohol*, 1940, **1**, 483.

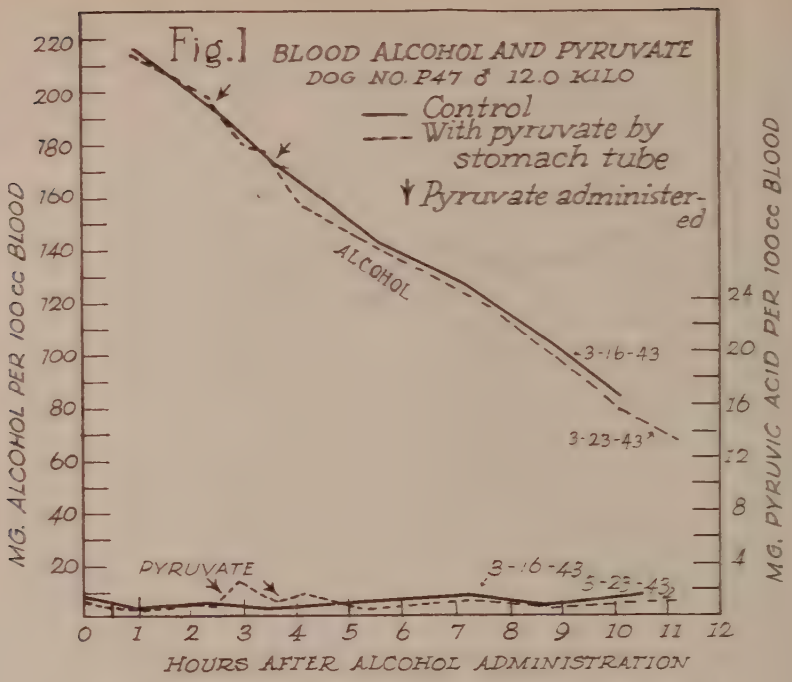
Fig. I BLOOD ALCOHOL AND PYRUVATE
DOG NO. P47 ♂ 12.0 KILO
Control
With pyruvate by stomach tube
Pyruvate administered
ALCOHOL
3-16-43
3-23-43
PYRUVATE
3-16-43
3-23-43
MG. ALCOHOL PER 100 cc BLOOD
MG. PYRUVIC ACID PER 100 cc BLOOD
HOURS AFTER ALCOHOL ADMINISTRATION

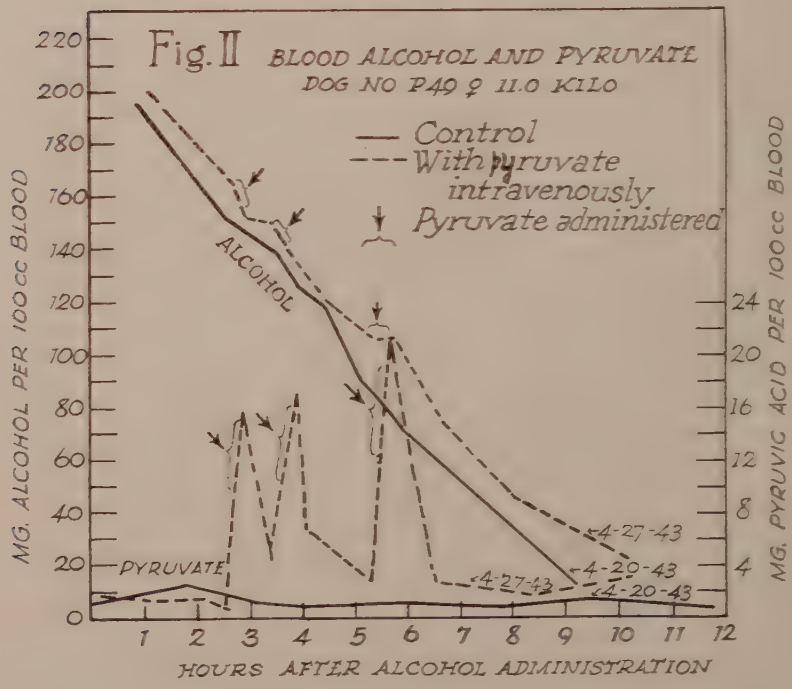
Fig. II BLOOD ALCOHOL AND PYRUVATE
DOG NO P49 ♀ 11.0 KILO
Control
With pyruvate intravenously
Pyruvate administered
ALCOHOL
4-27-43
4-20-43
4-27-43
4-20-43
PYRUVATE
MG. ALCOHOL PER 100 cc BLOOD
MG. PYRUVIC ACID PER 100 cc BLOOD
HOURS AFTER ALCOHOL ADMINISTRATION

Fig. II presents graphically the data obtained from following the rate of disappearance of alcohol alone and following the administration of a 10% solution of sodium pyruvate intravenously in 0.5 g per kilogram doses. It will be noted that 3 doses of sodium pyruvate intravenously over a period of 4 to 5 hours had no influence on the rate of metabolism of ethyl alcohol. The results shown in these two graphs are typical of those obtained with similar studies on 4 other dogs.

It is suggested that the results of Westerfeld, Stotz and Berg, which appear to indicate that pyruvate increases the rate of metabolism of ethyl alcohol, are due to the fact that absorption of alcohol from the stomach was not complete during the control period as was evidenced by the unusually low values for blood alcohol; and that following pyruvate administration, which may have retarded further absorption of alcohol by its hypertonicity, the true normal rate of alcohol disappearance was obtained. This is supported by the fact that the rate of disappearance of alcohol reported by these authors following pyruvate is almost identical with the rate of disappearance we have observed in our controls—*i.e.*, about 20 mg % per hour.

It is believed from our experience that the high levels of blood pyruvate reported by Westerfeld, Stotz, and Berg following sodium pyruvate by stomach tube may have been due to the struggling and other activity of the dogs. The low levels of blood pyruvate which they reported after ingestion of alcohol may, therefore, have been due to the decreased activity of the dogs as a result of the sedative action of alcohol and not to the increased utilization of the pyruvate in the metabolism of the alcohol as is thought by these workers.

Conclusions. Studies on 6 dogs show that the administration of sodium pyruvate by stomach tube or intravenously has no influence on the rate of metabolism of ethyl alcohol.

14372

The Action of Antibiotics on Organisms Producing Gas Gangrene.

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The bacteriostatic action of penicillin on the gas gangrene anaerobes and its efficacy in the treatment of experimental gas gangrene have been reported.[1-7] The present paper compares aspergillic acid[8-10] with other antibiotic agents and sulfonamides *in vitro* on *Cl. perfringens, Cl. septicum, Cl. oedematiens*, and a strain of *Cl. perfringens* made fast to sulfonamides by repeated transfer in broth containing sulfathiazole, and *in vivo* on *Cl. perfringens* infection in mice.

The medium used for the antibiotic tests *in vitro* contained 2% bacto tryptose, 0.2% glucose, 0.5% sodium chloride, 0.1% disodium phosphate, and 0.1% sodium thioglycollate; pH 7.4 to 7.6. Paraffin-vaseline seals were used on all cultures and

1 Chain, E., Florey, H. W., Gardner, A. D., Heatley, N. G., Jennings, M. A., Orr-Ewing, J., and Sanders, A. G., *Lancet*, 1940, **239**, 226.

2 Abraham, E. P., Chain, E., Gardner, A. D., Fletcher, C. M., Heatley, N. G., Jennings, M. A., and Florey, H. W., *Lancet*, 1941, **241**, 177.

3 Hobby, G. L., Meyer, K., and Chaffee, E., PROC. SOC. EXP. BIOL. AND MED., 1942, **50**, 277.

4 Florey, H. W., and Jennings, M. A., *Brit. J. Exp. Path.*, 1942, **23**, 120.

5 McIntosh, J., and Selbie, F. R., *Lancet*, 1942, **243**, 750.

6 McIntosh, J., and Selbie, F. R., *Lancet*, 1943, **244**, 793.

7 Hac, L. R., and Hubert, A. G., PROC. SOC. EXP. BIOL. AND MED., 1943, **53**, 61.

8 White, E. C., *Science*, 1940, **92**, 127.

9 White, E. C., and Hill, J. H., *J. Bact.*, 1943, **45**, 433.

10 Jones, H., Rake, G., and Hamre, D. M., *J. Bact.*, 1943, **45**, 461.

TABLE I.
Activity *in vitro* of Some Antibiotic Agents and Sulfonamides Against the Gas Gangrene Anaerobes.
Figures given are the smallest amounts completely inhibiting growth.

Substance tested	*Cl. perfringens* W*	*Cl. perfringens* ST fast	*Cl. septicum* *	*Cl. oedematiens* *
Penicillin M837-A 500 O.U./mg				
O.U/ml	0.15	0.135	0.075	0.025
μg/ml	0.3	0.27	0.15	0.05
Aspergillic Acid 6+15 256 R.U./mg				
R.U./mg†	10.2	7.6	10.2	5.1
μg/ml	40	30	40	20
Tyrothricin				
μg/ml	0.05		0.01	
Gliotoxin				
μg/ml	100			
Sulfathiazole				
μg/ml	25	>250		
Sulfadiazine				
μg/ml	7	>185		
Sulfanilamide				
μg/ml	150	>250		
Homosulfanilamide				
μg/ml	16	5		

* We are indebted to Drs. G. B. Reed and J. H. Orr for these cultures.

† R.U./mg were determined by comparison of the activity *in vitro* of this preparation with a standard preparation.[10]

tests with *Cl. oedematiens* and *Cl. septicum*.

In setting up the tests, decreasing amounts of broth solutions of the different antibiotic substances were placed in a series of 13 x 100 mm tubes, broth added to bring the volume in each tube to 3.8 ml, and the tubes placed in boiling water for 15 minutes. After cooling, 0.2 ml of the proper culture dilution to contain 5000-50,000 organisms was added to each tube as inoculum. In all tests control tubes containing no antibiotic substance were included. The tubes were incubated for 16-18 hours at 37°C and the smallest amount of antibiotic substance inhibiting growth, as indicated by lack of turbidity, was taken as the end-point. Penicillin and gliotoxin were added to the tubes after boiling, because both are unstable at high temperatures.

For the sulfonamide tests *in vitro*, freshly prepared MacLeod's medium,[11-12] containing 0.1% sodium thioglycollate and free from *p*-amino benzoic acid, was used. The sulfonamides were dissolved in M/15 phosphate buffer and sterilized by autoclaving. The technic of the tests was the same as above except that boiling was omitted. *Cl. perfringens* W and a sulfonamide-fast strain of *Cl. perfringens* were the only anaerobes which uniformly grew well in this medium. The results of these tests are given in Table I.

In repeated tests with tyrothricin, the minimum inhibiting concentrations varied from 0.6 to 0.01 μg per ml, indicating that this agent has about the same activity *in vitro* as the penicillin preparation tested.

Owing to the different types of media used, the sulfonamide tests and the antibiotic tests are not strictly comparable. The susceptibility of the sulfathiazole-fast strain of *Cl. perfringens* to the bacteriostatic action of the antibiotic agents was to be expected since it has been shown that sulfonamide-fast pneumococci are inhibited *in vitro* by penicillin.[13-14] Also of interest were the results of preliminary experiments with homosulfanilamide (4-amino methyl benzol sulfonamide),* which differs

[11] MacLeod, C. M., *J. Exp. Med.*, 1940, **72**, 217.

[12] MacLeod, C. M., and Mirick, G. S., *J. Bact.*, 1942, **44**, 277.

[13] Powell, H. M., and Jamieson, W. A., PROC. SOC. EXP. BIOL. AND MED., 1942, **49**, 387.

[14] McKee, C. M., and Rake, G., PROC. SOC. EXP. BIOL. AND MED., 1942, **51**, 275.

* This compound appears in the German literature under the name Marfanil. (Schreus, H. T., and Brauns, A., *Klin. Woch.*, 1941, **20**, 1233.)

from the other sulfonamides in not being inhibited by *p*-amino benzoic acid.

Albino mice weighing 20-23 g were infected with 0.05 ml of a 10^{-1} dilution of a 6-hour culture of *Cl. perfringens* W given intramuscularly in the thigh. The dilution was made in broth containing $CaCl_2$ so that each mouse received 5 mg of calcium chloride with the inoculum. This 6-hour culture, undiluted, contained 2 to 5 M.L.D.s of toxin per ml as determined by the lecithin test.† The mice were treated by intramuscular injection of 0.1 ml of the substance to be tested, given in the same area in the thigh as the infecting dose, either immediately following infection or one hour later.

The results of the experiments *in vivo* are given in Table II (immediate treatment) and Table III (delayed treatment). Since it is difficult to be sure that the drug is being given in exactly the same place as the infecting dose irregular results, such as appear in Table II, might be expected. Without the use of large numbers of animals it would not be possible to determine 50% protecting dose of any substance.

TABLE II.
Cl. perfringens Infection in Mice Treated Locally, at the Time of Infection, with Sulfonamides or Antibiotic Agents.

No. of mice	Treatment	Lived	Died
5	Sesame Oil	0	5
5	15 O.U. (0.1 mg) Penicillin*	5	0
5	1 mg Aspergillic Acid*	4	1
5	1 " Tyrothricin*	0	5
5	1 " Gliotoxin*	0	5
10	None	0	10
10	0.5 mg Aspergillic Acid	10	0
10	5 O.U. (0.033 mg) Penicillin	5	5
10	2.5 mg Sodium Sulfadiazine	10	0
10	None	0	10
10	0.2 mg Aspergillic Acid	3	7
10	0.1 " " "	2	8
10	None	0	10
10	0.25 mg Sodium Sulfadiazine	10	0
10	0.05 " " "	5	5
10	None	1	9
10	0.1 mg Sulfadiazine	10	0
10	0.05 " Sodium Sulfadiazine	7	3

* Suspended in 0.1 ml of sterile sesame oil. In other experiments solutions were used.

† We are indebted to Dr. W. L. Koerber of the Biological Laboratories of E. R. Squibb & Sons for the toxin assay. (Koerber, W. L., and Alture-Werber, A., *J. Immun.*, 1942, **45**, 223.)

TABLE III.
Cl. perfringens Infection in Mice Treated Locally, One Hour After Infection, with Sulfonamides, Antibiotic Agents, or Antitoxin.

No. of mice	Treatment	Lived	Died
10	None	0	10
10	10 O.U. (0.101 mg) Penicillin	10	0
10	5 O.U. (0.05 ") "	10	0
10	2 mg Sodium Sulfadiazine	9	1
10	0.2 " " "	10	0
10	10 Units Antitoxin	10	0
10	1 " "	8	2
10	None	0	10
10	2.5 O.U. (0.025 mg) Penicillin	10	0
10	0.5 O.U. (0.005 ") "	7	3
10	1.0 mg Sodium Sulfadiazine	9	1
10	0.05 " " "	1	9
10	1 Unit Antitoxin	7	3
10	0.1 " "	0	10

Experiments with guinea pigs infected via wounds by the method of Reed and Orr[15] were not successful due to the frequency of intercurrent infections and to the toxicity for guinea pigs of repeated doses of penicillin.[16]

Summary. 1. The antibiotic substances and sulfonamides arranged in the order of decreasing bacteriostatic properties *in vitro* for the anaerobes of the gas gangrene group are: Tyrothricin, penicillin, sulfadiazine, sulfathiazole, aspergillic acid, gliotoxin, and sulfanilamide.

2. Penicillin and aspergillic acid were active *in vitro* against a sulfathiazole-fast strain of *Cl. perfringens*.

3. When given locally to mice infected intramuscularly with *Cl. perfringens*, penicillin and sodium sulfadiazine were more effective than aspergillic acid. Gliotoxin and tyrothricin were not effective in doses of 1 mg suspended in sesame oil.

[15] Reed, G. B., and Orr, J. H., *War Med.*, 1942, **2**, 59.

[16] Hamre, D. M., Rake, G., McKee, C. M., and MacPhillamy, H. B., *Am. J. Med. Sci.*, 1943, **206**, 642.

14373

Prothrombin Time in Hypertensives with Special Reference to Cerebral Accidents.

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Recent work of Savacoll[1] and others has shown that the tendency for hemoptysis in pulmonary tuberculosis may be associated with prothrombin deficiency.

The present work was undertaken primarily to determine whether or not there is a relationship between the prothrombin time and cerebral accidents in hypertensives.

The general plan of experimentation was to study 2 approximately parallel groups of patients, one with and the other without cerebral accidents, and compare their prothrombin time under identical conditions.

Two groups of 8 male patients were selected from the inmates of the Home for Incurables. All the so-called complicated cases where the hypertension was associated with diabetes, lues, or some other extraneous factor were excluded from the study. Each patient was subjected to thorough physical examination which included urinalysis, hemogram, Wassermann and Kahn, as well as NPN. The blood pressure was taken at frequent intervals, and the accompanying tables give us the average of several readings. The prothrombin time was determined according to the method of Smith and blood was collected at approximately the same time of the day. Hemoglobin values were determined by Photo-Electric Colorimeter.

Summary and Conclusions. The results in the accompanying tables are self-explanatory and indicate that there is no clear relationship between cerebral accidents and the prothrombin time in hypertensives. The prothrombin time in hypertensives shows upper limits of normal values without any definite diagnostic significance.

[1] Savacoll, J. W., *Am. J. M. Sc.*, 1941, **201**, 830.

TABLE I.
Hypertensives Complicated by Cerebral Accidents.

	Age	Hgb.	RBC	WBC	NPN	Pro. time	B.P.
1	52	19.8	6.21	9.950	30.6	30	185/120
2	52	14.4	4.39	5.725	34.7	34	210/100
3	58	18.6	5.56	11.175	30.1	39	170/99
4	68	16.2	4.74	6.825	26.8	28	165/112
5	70	15.7	4.95	7.058	24.3	39	215/99
6	70	15.7	5.35	4.950	34.2	30	168/113
7	70	15.7	4.90	7.200	37.4	31	176/95
8	88	15.6	4.78	5.275	31.7	25	214/115

TABLE II.
Hypertensives Without Cerebral Accidents.

	Age	Hgb.	RBC	WBC	NPN	Pro. time	B.P.
1	53	17.6	5.65	5.100	33.6	34	180/124
2	58	14.4	4.59	5.075	27.1	30	165/95
3	62	16.3	5.71	6.850	31.8	29	200/123
4	66	15.8	4.71	5.474	30.9	33	192/135
5	70	17.1	5.84	5.250	29.5	36	162/110
6	72	16.3	5.18	7.800	36.2	31	143/101
7	76	12.9	4.13	5.350	49.6	28	155/135
8	77	15.2	4.60	6.475	26.3	31	159/104

Hgb.—Hemoglobin in g.
RBC—Erythrocytes.
WBC—Leucocytes.
NPN—Non-Protein Nitrogen.
Pro. Time—Prothrombin Time.
B.P.—Blood Pressure.

14374 P

Gravity Shock in Rabbits. I. Lack of Correlation Between Plasma Protein and Specific Gravity.

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The calculation of protein concentration from the specific gravity of human and dog serum or plasma[1,2] has been widely used in clinical and experimental laboratories especially since the development of the falling drop method for measuring specific gravity,[3,4] because it is quicker than analyses by the Kjeldahl method. It is important to know how the serum or plasma protein concentration changes in traumatic, hemorrhagic, and other kinds of shock, as well as in other pathological conditions, and much reliance has been placed on specific gravity determinations on the blood of such subjects.[5]

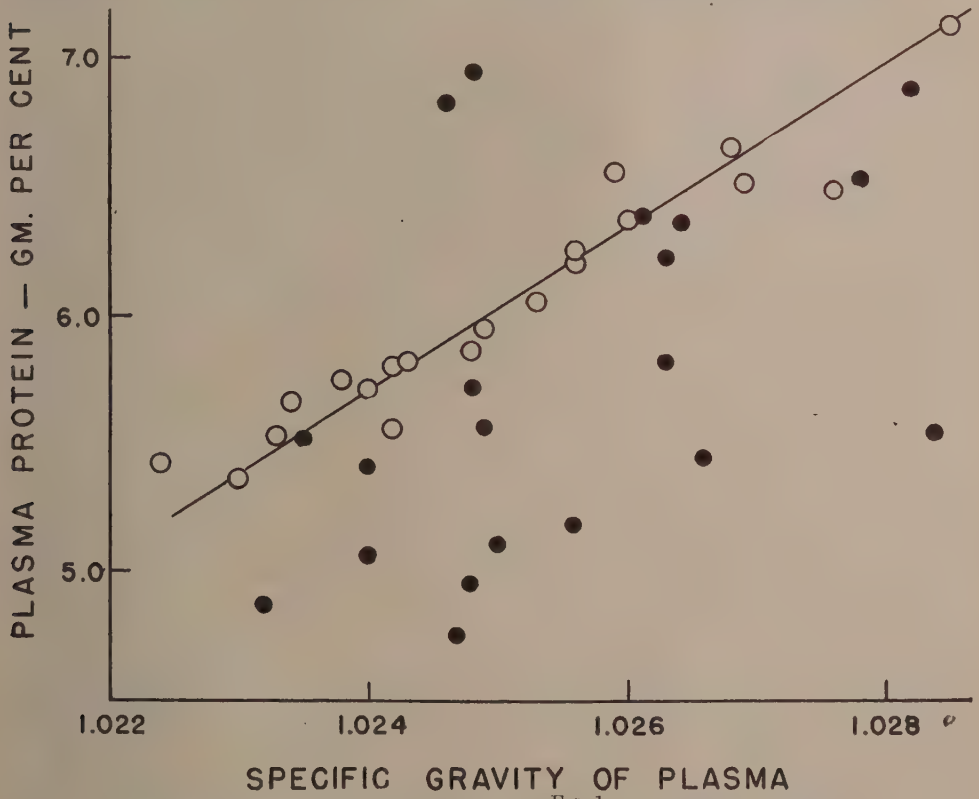

FIG. 1.

Concentration of protein (by Kjeldahl) in the plasma of normal rabbits (open circles) and of the same rabbits in gravity shock (solid circles) plotted against plasma specific gravity. Equation of line fitted to normal values is $P = 325\ (G_p - 1.0064)$.

[1] Moore, N. S., and Van Slyke, D. D., *J. Clin. Invest.*, 1929-30, **8**, 337.

[2] Weech, A. A., Reeves, E. B., and Goetsch, E., *J. Biol. Chem.*, 1936, **113**, 167.

[3] Barbour, H. G., and Hamilton, W. F., *J. Biol. Chem.*, 1926, **69**, 625.

[4] Guthrie, C. C., *J. Lab. and Clin. Med.*, 1931-32, **17**, 1158.

[5] Drew, C. R., Scudder, J., and Papps, J., *Surg., Gyn. and Obst.*, 1940, **70**, 859.

In order to determine whether the same relationship between specific gravity and protein concentration exists in rabbit plasma as in human and dog plasma, blood from 20 normal rabbits fed on Purina chow and later from the same animals suspended by their ears until they became unconscious from peripheral circulatory deficiency ("gravity shock")[6] has been studied. The plasma of arterial heart blood was analyzed for protein by the micro-Kjeldahl method, for glucose by the Folin-Wu method as modified by Andes and Northup,[7] and the specific gravity was determined by the falling drop method.

In Fig. 1 plasma protein before and during gravity shock is plotted against plasma specific gravity. The data for normal animals are well described by the line drawn through the open circles having the equation: $P = 375\ (G_p - 1.0064)$, which is of the same order of magnitude as the equation reported for human[1] and dog[2] plasma. For rabbits in gravity shock, however, the constant relation between specific gravity and protein concentration did not hold, as indicated by the solid circles, showing even greater divergencies than those found by Moore and Van Slyke[1] in human nephritic patients. The greatest differences below the line occurred in animals with high plasma glucose, as might be expected from the work of Simeone and Sarris.[8] More data are needed to determine why the discrepancies exist, although there is indication that increased glucose and non-protein nitrogen are involved.

It is concluded that the linear relationship between protein concentration and specific gravity of normal rabbit plasma is essentially the same as that for human and dog plasma. For rabbits in gravity shock, however, the correlation of protein concentration and specific gravity is low. A similar low correlation may be found for other animals showing shock symptoms.

[6] Allison, J. B., Cole, W. H., Leathem, J. H., Nastuk, W. L., and Anderson, J. A., *J. Biol. Chem.*, 1943, **147**, 255.

[7] Andes, J. E., and Northup, D. W., *J. Lab. and Clin. Med.*, 1938-39, **24**, 529.

[8] Simeone, F. A., and Sarris, S. P., *J. Lab. and Clin. Med.*, 1940-41, **26**, 1046.

14375

Pharmacology of Sodium Sulfanilylsulfanilate.*

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Sodium sulfanilylsulfanilate, a derivative of sulfanilamide, was first used therapeutically by Dochez and Slanetz[1] against a fatal infection in ferrets supposed to be due to and resembling dog distemper. MacIntyre and Montgomerie[2] in England employed this drug unsuccessfully in infections due to the distemper virus described by Carre-Laidlaw-Durkin. Oakley[3] used it without benefit in experimental influenza. Coggeshall[4] tested sodium sulfanilylsulfanilate in avian malaria. Hebb, Sullivan, and Felton[5] recommended

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† Deceased November 9, 1943.

[1] Dochez, A. R., and Slanetz, C. A., *Science*, 1938, **87**, 142.

[2] MacIntyre, A. B., and Montgomerie, R. F., *Brit. Med. J.*, 1938, **4032**, 875.

[3] Oakley, G. L., *Brit. Med. J.*, 1938, **1**, 895.

[4] Coggeshall, L. T., PROC. SOC. EXP. BIOL. AND MED., 1938, **38**, 768.

[5] Hebb, A., Sullivan, S. G., and Felton, L. D., *U. S. Pub. Health Rep.*, 1939, **54**, 1750.

TABLE I.
Fate of a 5-g Oral Dose of Sodium Sulfanilylsulfanilate.

Blood:
Blood was drawn 5 min, 1 hr, 6 hr and 24 hr after administration.
Blood contained only traces of the drug.
Urine and Feces:
Sulfanilylsulfanilate in mg; Output in Urine and Feces.

	4 hr	6 hr	24 hr	48 hr	72 hr	Total recovery	% recovery
Urine:							
Free	114	38	14	trace		166	3.3
Conjugated	27	21	11	"		59	1.2
Total	141	59	25			225	4.5
Feces:							
Free			1200	7	41	1248	24.9
Conjugated			700	2	31	733	14.6
Total			1900	9	72	1981	39.5

Percent recovery combined—44.0.

sodium sulfanilylsulfanilate and sodium sulfanilate for the treatment of lymphopathia venereum. This report deals with the pharmacology and toxicity of sodium sulfanilylsulfanilate.

Sodium sulfanilylsulfanilate was determined by the Marshall[6] method using alpha dimethylnaphthylamine in alcoholic solution as the coupling component. Determinations were made on whole blood, urine, and fecal discharges. The fate of single and repeated doses were studied in some normal subjects and in patients, following oral and intravenous administration.

Six subjects were given single 5 g doses of sodium sulfanilylsulfanilate. In all 6 cases only traces were observed in the blood. The drug, however, may be recovered in the urine and feces. As shown in Table I, only 4.5% of the total ingested was excreted in the urine while the fecal excretion was 39.5%. In 10 patients repeated oral doses of 5 g statim and 1 g every 4 hours yielded only traces in the systemic circulation. In 2 subjects 10 g 3 times daily resulted in systemic blood levels of 1.0 mg % per 100 ml or less.

When 50 ml of a 10% solution of sodium sulfanilylsulfanilate was administered intravenously immediate high blood concentrations were observed. However, as is usual with intravenous sulfonamide therapy, though the rise in concentration is rapid, the fall is equally fast. It will be noted in Chart II that 15 minutes following a 5 g intravenous administration the concentration in the blood was 12 mg per 100 ml. Five hours afterwards, it was only 1.0 mg per 100 ml of blood. This rapid fall was also observed by Dochez and Slanetz.[1] After intravenous administration most of the drug is recovered from the urine and only small amounts are present in the feces and is completely excreted in 24 to 48 hours. From 75 to 83% was recovered from the urine and feces. The urine alone accounted for 70 to 80% of the recovery. From 15% to 35% of the administered drug was conjugated.

The low recovery from the urine and the high fecal value, when the drug is given orally, might indicate that little of the drug is absorbed from the gastrointestinal tract. We administered orally to 6 rabbits 1 g per kilo body weight of this compound. Two of these rabbits received 1 g of sodium sulfanilylsulfanilate per kilo body weight for 4 days and 2 g for 2 subsequent days. Daily bloods drawn from the ear showed only traces of the drug. At the completion of the drug administration these animals were operated and specimens of venous blood, portal blood, and gall bladder bile were taken for analyses. The venous blood yielded only traces of the drug. The concentrations of the bloods obtained from the portal veins were 1.8 mg % and 2.0 mg % per 100 ml and from the bile the concentrations were 2.9 mg % and 7.2 mg % per 100 ml in 2 rabbits. Drains were inserted into the gall bladders and the ob-

[6] Marshall, E. K., Jr., *J. Biol. Chem.*, 1937, **122**, 263.

TABLE AND CHART II.
SULFANILYLSULFANILATE IN BLOOD, URINE AND FECES

[MGS.] SULFANILYLSULFANILATE OUTPUT IN URINE						RECOVERY	
HOURS	2	4	24	48	72	TOTAL	PER-CENT
FREE	1947	570	58	103	48	2726	54.5
CONJUGATED	973	330	11	20	12	1346	26.9
TOTAL	2920	900	69	123	60	4072	81.4

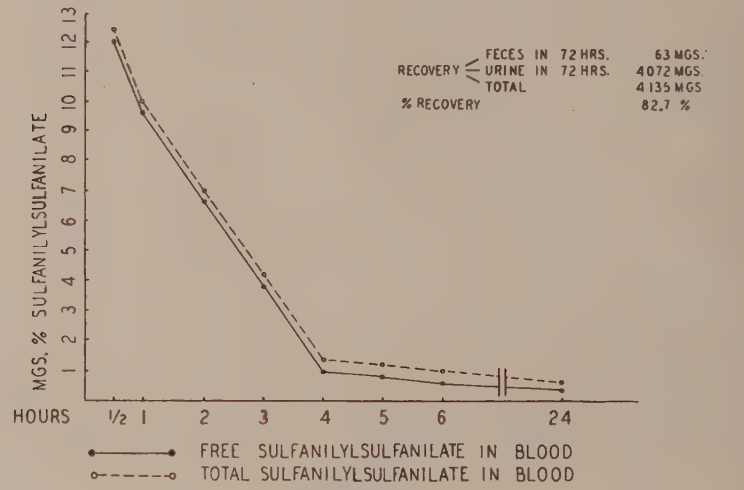

TABLE III.
Mg of Sulfanilylsulfanilate per 100 g of Material.

Rabbit No.	Ear blood	Portal vein blood	Bile	Stomach contents	Duodenal contents	Large gut contents
1	trace	2.0	7.8	22.6	1.0	
2	0.6	1.1	4.6	36.5	14.0	320.0
3	1.6	1.6	4.8	27.0	10.7	240.0
4	trace	2.1	5.9	39.1	9.4	290.0

servations were continued. Twenty-four hours after the drains were inserted ear blood showed traces and the drained bile 1.0 mg % per 100 ml of the drug. At this time the rabbits were given 1 g of sodium sulfanilylsulfanilate and 24 hours after the concentrations in the ear blood were 0.5 mg % per 100 ml and 0.6 mg % per 100 ml. For the bile the concentrations were 3.2 mg % per 100 ml and 4.3 mg % per 100 ml. Four rabbits were fed sodium sulfanilylsulfanilate and were sacrificed. Contents of various organs were analyzed for the drug (Table III). The greatest concentrations were found in the bile obtained from the gall bladder. This suggests that most absorption occurs by the portal system and may account for high concentrations in the feces as well as for its beneficial effect on lesions in the bowel as reported elsewhere.[7]

Discussion. Only traces of sodium sulfanilylsulfanilate were found in the blood when 0.5 to 5.0 g were administered orally. After 10 g 3 times daily the systemic blood levels were low. High systemic blood concentrations may be noted when the drug is given intravenously, however, the concentrations thus obtained fall very rapidly. In rabbits high concentrations were noted in the bile indicating portal absorption and excretion into the gastrointestinal tract.

[7] Levy, J., Holder, E., and Bullowa, J. G. M., *Am. J. Dig. Dis.*, 1942, **9**, 237.

14376

Cystine Content and Enzyme Digestibility of Powdered Hoof Proteins.

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Routh and Lewis[1] first described the effect of subdivision in enhancing the digestibility of keratin by proteolytic enzymes. Recently Wagner and Elvehjem[2] obtained growth in rats and chicks with powdered swine hoofs as the main source of protein. Their preparations must therefore have been susceptible to enzymatic digestion *in vivo*. The results obtained in the experiments described in this paper show that (a) the major part of cattle and horse as well as hog hoof powders are digestible by proteolytic enzymes *in vitro*, (b) the undigested residue contains considerably more cystine than does the total hoof protein, and (c) there is an inverse relationship between cystine content and digestibility by pancreatin in different parts of the individual cattle hoofs.

Cattle hoofs were washed with water and then were treated in various ways to reduce them to powder. In one instance, soaked hoofs were shredded with a coarse rasp and the raspings were run through a Premier colloid mill in the presence of large amounts of water, allowed to settle, filtered, washed, dried at 70°, and sieved. For most preparations the cleaned hoofs were dried at 70° for several days until they became brittle, then broken to small pieces by pounding in an iron vessel and finally ground in a porcelain ball mill for 24 to 48 hours. It should be mentioned that the mild grinding procedures differentiate these experiments somewhat from those described by Routh,[3] who was able to make wool and chicken feathers nutritionally available by grinding them for several weeks in a large iron mill equipped with steel balls.

Portions of the powdered cattle hoof were incubated with solutions of pancreatin, pepsin, papain, ficin, and a bacterial protease, after adjustment of the pH to the optimal for the particular enzyme. A considerable proportion of the hoof was digested in each case, showing that, in general, cattle hoof can be digested by proteolytic enzymes. Pancreatin was used in most of the subsequent experiments.

In order to study the influence of particle size on digestibility by pancreatin, various mesh sizes of cattle, horse, and hog hoof powders were treated separately. The results are shown in Table I.

TABLE I.

Effect of Size of Particle on Digestibility of Hoof Preparations* by Pancreatin.†

Mesh size	Cattle hoof	Horse hoof	Hog hoof
	Soluble N as percentage of total N.‡		
40-60	—	8	37
60-80	17	11	46
80-100	26	13	55
100-140	30	18	59
140-200	33	24	65
>200	52	39	81

* Dried at 70°, broken and ball-milled.

† 2 g keratin, 5 ml pancreatin solution (4.4 mg N per ml), 45 ml 1% $Na_2HPO_4 \cdot 10H_2O$, shaken at room temperature for 24 hours. The final pH varied from 6.5 to 7.5.

‡ Not corrected for the amount of N soluble in the buffer alone. Except with hog hoof, this was a negligible factor. 5% of the 80-100-mesh and 10% of the 200-mesh hog hoof powder nitrogen was soluble in the buffer only.

Samples of 200-mesh cattle, horse, and hog hoof preparations were subjected to exhaustive proteolysis by incubation with several changes of pepsin and then pancreatin, each time for 48 hours at 37°. The residues were washed and dried and analyzed for H_2O, N, S, ash, and total cystine content.[4] The results, corrected for moisture and ash, are shown in Table II.

[1] Routh, J. I., and Lewis, H. B., *J. Biol. Chem.*, 1938, **124**, 725.

[2] Wagner, J. R., and Elvehjem, C. A., Proc. Soc. Exp. Biol. and Med., 1942, **51**, 394; *Poultry Sci.*, 1943, **22**, 275.

[3] Routh, J. I., *J. Biol. Chem.*, 1940, **135**, 175; *J. Nutrition*, 1941, **23**, 125; 1942, **24**, 399.

[4] Mecham, D. K., *J. Biol. Chem.*, in press.

TABLE II.
Analyses of Hoof Powders and Residues from Enzyme Digests.*

	Yield,† %	N	S	Total cystine‡
Cattle hoof—original	—	16.2	2.2	5.9
residue	19	15.9	3.0	9.0
Horse hoof —original	—	16.3	2.3	5.9
residue	28	15.6	3.4	8.4
Hog hoof —original	—	15.6	1.5	4.4
residue	2.5	12.8	2.3	9.0

* Corrected for moisture and ash.
† The yield results are only approximate since unavoidable losses occurred during handling.

The undigested portion in each case was considerably richer in cystine than the original.

It is well recognized that the physical structure of the cattle hoof is not uniform. Those portions comprising the bottom and rear of the hoof (subunguis) become soft when wet and can readily be pared with a sharp knife, in contrast to the front jutting portion (unguis), which is considerably harder. The hard and soft parts of several hoofs were separated, dried, ground, and analyzed for cystine and for digestibility by pancreatin. The hard portion contained 5.6% cystine, and 51% of the insoluble nitrogen was changed into a soluble form by pancreatin in 24 hours at 37°. The soft portion contained only 2.8% cystine, and 71% of the nitrogen was digested under the same conditions.

Thus, as would be expected from the anatomical structure of the hoof, the powdered hoof material is a mixture of at least 2 and possibly many proteins containing differing numbers of disulfide linkages. The greater number of disulfide cross-links in the unguis might be pictured as contributing to the hoof protein structure the physical stability necessary for resistance to abrasion and shock.

Probably only those fractions resistant to proteolytic enzymes and rich in cystine should be regarded as keratins.

The importance of the disulfide linkage to the enzyme resistance of wool has been described by Geiger, Patterson, Mizell, and Harris.[5] When the wool fibers were reduced by thioglycolic acid they were readily attacked by proteases. The correlation between cystine content and enzymic resistance in hoof proteins is interpretable in the light of their results.

I am indebted to the following persons in this laboratory: C. D. Anselm for technical assistance, D. K. Mecham for the cystine analyses, L. Kline for the sample of bacterial protease, and H. Lineweaver for helpful suggestions.

Summary. Considerably more than half of dried powdered cattle, horse, and hog hoof material was found to be digestible by proteolytic enzymes. Hog hoof proved the most readily digestible and horse hoof the least. The undigestible residue in each case contained more cystine than the original preparations. The soft part of the cattle hoof contained less cystine and was more readily digested than the horn-like front portion. Thus hoof powder consists of at least two and possibly a number of different kinds of proteins.

[5] Geiger, W. B., Patterson, W. I., Mizell, L. R., and Harris, M., *J. Res. Nat. Bur. Stand.*, 1941, **27**, 459.

14377

Renin Substrate and Angiotonase in Dogs' Lymph and Plasma.*

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Besides renin, two substances of protein nature appear to be primarily responsible for the regulation of the angiotonin (hypertensin) concentration in the blood: Renin substrate (hypertensinogen) and angiotonase (hypertensinase), precursor and inactivator respectively of angiotonin.[1,2,3] Both have been demonstrated in the blood of dogs but not as yet in other body fluids. The present communication deals with the occurrence of these two substances in lymph.

Methods. Lymph was obtained from the cervical lymph ducts of normal dogs anesthetized with sodium pentobarbital. From the same animals, blood was taken simultaneously for preparation of plasma samples. Heparin was used as anticoagulant.

For determination of renin substrate content, lymph and blood plasma samples were incubated in quantities varying from 0.1 to 1.0 cc with a constant amount of renin for 10 minutes at room temperature (approximately 20 to 28°C), and the vasoconstrictor activity of the angiotonin formed was measured using the Läwen-Trendelenburg toad preparation. In order to insure a complete conversion of the renin substrate into angiotonin, an excess of renin was used. It was assumed that the amount of angiotonin so formed was proportional to the quantity of its precursor, the renin substrate originally present. In preliminary experiments, the quantity of renin was determined, which, when incubated with 1.0 cc of plasma, produced a maximal vasoconstrictor effect. A fourfold amount was adopted as a standard quantity in the substrate assays.

For determination of angiotonase, angiotonin was incubated with 1.0 cc of lymph or plasma for one hour at 37°C. Care was taken to avoid hemolysis of the blood, in order to exclude contamination of the samples with angiotonase derived from red cells. As observed by Fasciolo *et al.*,[3] the latter contain approximately 100 times as much angiotonase as plasma. Since practically no renin is present under such conditions, it was possible to estimate the quantity of angiotonase from the amount of angiotonin inactivated during incubation. Thus, angiotonin activity was used as an indirect criterion of the concentration of both renin substrate and angiotonase.

For estimation of the angiotonin, the samples were diluted immediately after incubation to 100 cc with Ringer's solution containing 0.5% sodium citrate. Their vasoconstrictor activity was then tested on the toad preparation which was perfused with the same modified Ringer's solution. Neither lymph nor plasma alone, in the dilutions used, produced any significant change in the rate of flow. In order to avoid effects due to differences in the responsiveness of individual toads, lymph and plasma samples from each dog, along with a suitable control, were always assayed in the same toad. The order was varied to offset any effects of decreasing responsiveness in the toad preparation.

The renin preparation was extracted from acetone-treated hog kidney cortex with $NaCl/NaHCO_3$ according to the Wakerlin and

* Aided by a grant from the Dazian Foundation for Medical Research.

† Present address: Institute of Experimental Biology, University of California, Berkeley, Calif.

1 Kohlstaedt, K. G., Helmer, O. M., and Page, I. H., PROC. SOC. EXP. BIOL. AND MED., 1938, **39**, 214.

2 Braun-Menendez, E., Fasciolo, J. C., Leloir, L. F., and Munoz, J. M., *Rev. Soc. Argent. de Biol.*, 1939, **15**, 420.

3 Fasciolo, J. C., Leloir, L. F., Munoz, J. M., and Braun-Menendez, E., *Rev. Soc. Argent. de Biol.*, 1940, **16**, 643.

TABLE I.
Renin Substrate in Lymph and Plasma.
Vasoconstrictor Effects after Incubation with Renin.

Amount body fluid* cc	No. of toads	% change in flow (toad perfusion) Mean value and standard error† A Lymph* + renin	B Plasma* + renin	C Renin control	Difference (A-B) and standard error†
1.00	7	—71 ± 3	—68 ± 5	— 1 ± 4	— 3 ± 6
0.50	7	—63 ± 5	—64 ± 6	+ 1 ± 10	+ 1 ± 8
0.20	8	—40 ± 4	—57 ± 4	+13 ± 4	+17 ± 6
0.15	3	—36 ± 2	—38 ± 7	+11 ± 2	+ 2 ± 7
0.10	7	—32 ± 5	—32 ± 5	+ 7 ± 7	0 ± 7

* Samples obtained from 12 dogs.

† Standard error of the mean (S_x) and standard error of the difference of the means (S_D) computed for small samples:[7] $S_x = \sqrt{\frac{\Sigma(x^2)}{N(N-1)}}$; $S_D = \sqrt{\frac{\Sigma(x_1^2) + \Sigma(x_2^2)}{(N_1-1) + (N_2-1) \times \frac{N_1 \times N_2}{N_1 + N_2}}}$

The difference (A — B) is considered significant only when $\geqq 3 \times S_D$.

TABLE II.
Angiotonase in Lymph and Plasma.
Vasoconstrictor Effects of Angiotonin after Incubation with These Fluids.

Fluids incubated	No. of toads	% change in flow (toad perfusion) Mean and standard error	Difference (A-B) and standard error
A: Angiotonin + lymph*	12	—38 ± 5	—24 ± 6
B: Angiotonin + plasma*		—14 ± 3	
C: Angiotonin + control*		—71 ± 2	

* Samples obtained from 6 dogs.

Johnson modification of Grossman's method.[4,5] For each renin substrate assay, 0.2 cc of a standard renin solution containing 1.2 mg nitrogen per cc was used. Five cc of this solution produced a rise in blood pressure of 60 mm Hg. in 2.5 minutes, when injected intravenously into a normal unanesthetized dog.

Purified angiotonin preparations containing approximately 5 units per cc,[6] were most kindly supplied by Dr. I. H. Page. In each angiotonase assay, the smallest quantity of angiotonin was used which, when tested alone, produced a decrease in flow of from 60 to 80%.

Results. The experimental data obtained for renin substrate are summarized in Table I. For a given dose level, lymph and plasma, after incubation with renin, produced practically the same degree of vasoconstriction. With decreasing concentrations the effect declined at the same rate for both fluids. If it be assumed that in lymph the same mechanism is involved in causing the observed vascular effects as in plasma, it is apparent that renin substrate is present in similar concentrations in both.

However, the concentrations of angiotonase were found to be considerably different in these body fluids. Table II indicates that 1.0 cc of plasma contained enough angiotonase to inactivate most of the angiotonin present. Under identical conditions, 1.0 cc of lymph, on the other hand, neutralized a considerably smaller fraction of the angiotonin, the pressor action being reduced only to approximately one-half of that of the angiotonin control. If the inactivation of

4 Wakerlin, S. E., and Johnson, C. A., PROC. SOC. EXP. BIOL. AND MED., 1941, **46**, 104.

5 Grossman, E. B., PROC. SOC. EXP. BIOL. AND MED., 1938, **39**, 40.

6 Page, I. H., personal communication.

7 Arkin, H., and Colton, R. R., *Outline of Statistical Methods*, New York, 1939, 126.

angiotonin by lymph and plasma involves the same mechanism, it is evident that the concentration of angiotonase in lymph is considerably lower than in plasma. It is not known whether the relatively much higher angiotonase content of the red blood cells[3] is responsible for the higher concentration of this substance in plasma, as compared to lymph.

Summary. Cervical lymph and plasma from normal dogs were compared as to their contents of renin substrate and angiotonase. It was observed that renin substrate occurs in similar concentrations in both lymph and plasma, but that the quantity of angiotonase present in lymph is considerably less than that in plasma.

The authors are grateful to Eleanor Kruger and Helen Mendelson for their excellent assistance.

14378

Action of Testosterone and Prolactin on the Corpora Lutea of the Rat.*

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In previous studies[1,2] it was shown that under certain experimental conditions the continuous administration of testosterone propionate (total dose, 20 to 40 mg) to female rats led to the formation of hypertrophied corpora lutea such as normally occurs during pregnancy. These structures likewise were found to have an active function, since deciduomata could be produced in the uteri while they were present. At that time we felt that the hypertrophy and function of these corpora resulted from an excessive secretion of prolactin due to a direct effect of the testosterone on the anterior hypophysis.

The present report deals with a series of experiments designed to determine (1) whether large dosages of prolactin lead to hypertrophy of corpora lutea, and (2) whether this luteal hypertrophy is necessary for active function, as indicated by the production of deciduomata.

Fifty-nine adult female rats of the Stanford strain, averaging 110 days of age, were employed. Daily vaginal smears of each animal were studied for 28 days previous to injection, and the first injection was always given during late estrus. The injections were administered subcutaneously for a period of 10 days. The testosterone propionate† was dissolved in sesame oil in varying concentrations and given in single daily doses of from 0.1 cc to 0.05 cc, according to the total dosage indicated in Table I. The prolactin was made up in saline solutions of varying concentrations and from 2 to 4 injections were given daily, but each consisted of 0.2 cc. The control animals received equivalent amounts of sesame oil or saline solution.

On the 5th day the animals were anesthetized, the abdomen opened, and a silk thread introduced for a distance of about 1 cm through the lumen of one of the uterine horns. The rats were sacrificed on the 11th day and the ovaries, together with sections of the uteri taken at the level of the traumatization with

* Supported in part by the Rockefeller Fluid Research Fund of Stanford University School of Medicine. Our thanks are due to Mr. V. Montesclaros and Mrs. Agnes Chinn for their technical assistance.

1 Fluhmann, C. F., and Laqueur, G. L., *Endocrinology*, 1942, **31**, 375.

2 Laqueur, G. L., and Fluhmann, C. F., *Endocrinology*, 1942, **30**, 93.

† Our thanks are due to the Ciba Pharmaceutical Products, Inc., Summit, N.J., for the testosterone propionate (Perandren-Ciba) and to the Schering Corporation, Bloomfield, N.J., (Lactogenic Hormone-Schering) used in this study.

TABLE I.

Effect of Graded Dosages of Prolactin and Testosterone Propionate on the Size of Corpus Luteum Cells and Corpus Luteum Function.

Exp.	No. rats	Material injected	Total dosage	Size of corpora lutea		Deciduoma reaction	
				Normal	Hypertrophied	Negative	Positive
I	5	Testosterone	1.0 mg	5	0	5	0
II	5	"	2.5 "	5	0	3	2
III	6	"	5.0 "	4	1 (slight)	0	6
IV	5	"	10.0 "	5	0	0	5
V	4	"	20.0 "	0	4	0	4
VI	5	Prolactin	600.0 I.U.	5	0	1	4
VII	3	"	1200.0 "	3	0	0	3
VIII	2	"	2400.0 "	2	0	0	2
IX	3	Prolactin	400.0 "	0	3	0	3
		Testosterone	5.0 mg				
X	4	Prolactin	600.0 I.U.	0	4	0	4
		Testosterone	20.0 mg				
XI	6	Saline	1.0 cc	6	0	6	0
XII	11	Sesame Oil	0.5 "	11	0	11	0

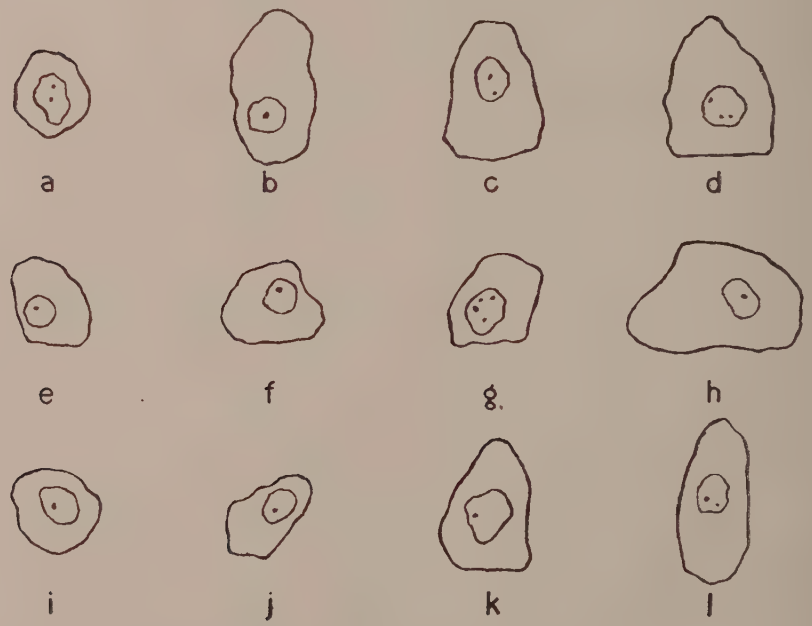

FIG. 1.

Camera Lucida Tracings of Corpora Lutea Cells.

(a) During diestrus
(b) Pregnancy, 16th day
(c) " 18th "
(d) Day of littering
(e) After 600 I.U. of prolactin
(f) " 1200 " " "
(g) " 2400 " " "
(h) " 400 " prolactin and 5 mg testosterone
(i) " 5 mg of testosterone
(j) " 10 " " "
(k) " 20 " " "
(l) " 20 " " "

the thread, were fixed and stained for histologic study.

The results are indicated in Table I. It is seen that a total dosage of 1 mg of testosterone propionate given under these conditions failed to produce either hypertrophy of the corpora lutea or deciduomata. Deciduomata were formed in 2 of 5 animals given 2.5 mg and in all animals receiving 5 or 10 mg, but hypertrophy of the luteal cells was found only when 20 mg of the hormone were given. This enlargement is illustrated by the representative camera lucida drawings in Fig. 1, where it also can be compared with cells of various other types of corpora lutea.

Uniform results were obtained with prolactin. With 600, 1200, and 2400 I.U., the uteri reacted with deciduomata in 9 out of 10 rats but in no instance was there any enlargement of the corpora.

It is significant that the combined administration of 5 mg of testosterone propionate and 400 I.U. of prolactin produced enlarged corpora, because at this dosage level neither hormone by itself produced this effect.

Summary. The administration of testosterone to rats, provided the initial injection is given during late estrus, leads to an enhanced and prolonged function of the corpora lutea, so that deciduomata can be produced by traumatization of the uterus. The minimal total dosage necessary to produce this effect is between 2.5 and 5.0 mg, given over a period of 10 days. If it is increased to 20 mg there is, in addition, a marked hypertrophy of the corpora lutea cells, similar to that of normal pregnancy.

A similar administration of prolactin in total dosages of from 600 to 2400 I.U. produces active corpora lutea, as shown by deciduoma formation, but there occurs no hypertrophy of the luteal cells.

14379 P

Inhibition of Experimental Liver Cancer in Rats by Addition of an Adsorbent to the Diet.

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The inhibiting influence of yeast, liver, rice bran and their extracts on the carcinogenicity of the azo dyes has been studied recently by Sugiura and Rhoads[1] who reported that only part of the protective factor in yeast could be extracted with ether. We have observed that the relatively indigestible fractions of the foods mentioned, when placed in an aqueous solution of the carcinogenic dye known as butter yellow (paradimethylaminoazobenzene), adsorb considerable amounts of the dye *in vitro*. It therefore occurred to us that part of the protective influence of these dietary constituents on butter yellow cancer might be due to reduced absorption of the carcinogen and that the incidence of this type of experimental tumor might be greatly reduced by the inclusion in the diet of a good, non-toxic, indigestible adsorbent, such as the clay, montmorillonite.*

The present report deals with the adsorption capacity for butter yellow of several materials which might be suitable for inclusion in experimental diets containing this carcinogen, together with preliminary results on feeding one of these (montmorillonite) on the incidence and course of butter yellow induced tumors.

The adsorption capacity of the various materials for butter yellow, determined by the

[1] Sugiura, K., and Rhoads, C. P., *Cancer Research*, 1941, **1**, 3.

* Montmorillonite ($(Mg, Ca)O \cdot Al_2O_3 \cdot 5SiO_2 \cdot nH_2O$) is a natural white clay used by refiners for removing undesirable substances from foods and petroleum products. That used by us was mined in Gonzales County, Texas, and generously donated by the Milwhite Company of Houston, Texas.

general method of Fantus[2] for basic dyes, is expressed in milligrams of dye adsorbed by 10 g of the water insoluble material before and after subjection to 12 hours of peptic and 18 hours of tryptic digestion at 37°C.

	Before digestion mg	After digestion mg
Dried brewer's yeast	8.61	7.54
Dried beef liver	8.74	8.50
Rice bran	9.05	8.40
Unpolished rice flour	5.26	4.00
Montmorillonite	954.00	472.00

Since montmorillonite is practically insoluble except in strong NaOH or HFl solutions, the effect of digestion on its adsorption capacity is probably due to initial saturation of its surface with enzymes or other protein-like substances in the digestive fluids.

The feeding experiments were carried out on 40 young rats of the Wistar strain which were divided into 2 groups similar in number, sex, age, and weight. The control group was fed butter yellow according to the method used in the experiments of Sugiura and Rhoads.[1] The treated group received a diet containing 10 parts of the basic carcinogenic ration and 1 part (by weight) of montmorillonite. This adsorbent clay was assayed for its riboflavin content by the method of Swaminathan[3] and found to be free of this vitamin, an important feature since the protective effect of yeast, bran, and liver has been attributed partly to the high riboflavin content of these foods.[4]

During the period from the 60th to the 65th day of dieting, the animals of each group were placed in individual metabolism cages and the amount of butter yellow excreted in the urine was determined by colorimetric method. It was found that the animals receiving montmorillonite in their ration excreted about 53% less butter yellow in the urine than did the rats receiving none of the added adsorbent.

The results of feeding the carcinogenic diets for a period of 175 days are as follows:

1. Eighteen of the 20 control animals developed tumors which were capable of detection by palpation at the end of 175 days or by macroscopic examination at autopsy before this time. Only one of the 20 montmorillonite-fed rats developed a palpable tumor within the 175 days.

2. While only 8 of the 20 control rats survived the 175 days, 17 of the 20 montmorillonite-fed animals survived this period.

3. The average weight gain per animal during the first 100 days of the experiment was 74 g for the control group and 54 g for the montmorillonite-fed group.

4. The eyes, hair, and activity of the rats revealed no marked vitamin deficiency in either group.

Summary. The relatively indigestible fractions of yeast, liver, and rice bran efficiently adsorb butter yellow (paradimethylaminoazobenzene) and thus remove this carcinogen from solution *in vitro*. In order to determine whether an adsorbent alone might confer protection against carcinogenesis, a riboflavin-free adsorbent clay, montmorillonite, was added to a diet containing butter yellow and fed to 20 young rats. During 175 days of feeding, only one of the 20 animals developed a palpable liver tumor, whereas 18 of 20 control rats developed such a tumor. That a considerable portion of the carcinogen was eliminated by adsorption upon the clay was indicated by the observation that the rats receiving the clay excreted 53% less carcinogenic dye in the urine than did the animals receiving no montmorillonite.

[2] Fantus, B., *J. A. M. A.*, 1915, **64**, 1838.

[3] Swaminathan, M., *Ind. J. Med. Res.*, 1942, **30**, 23.

[4] Kensler, C. J., Sugiura, K., Young, N. F., Halter, C. R., and Rhoads, C. P., *Science*, 1941, **93**, 308.

14380

Reproduction on Purified Rations Containing Sulfaguanidine.

B. H. Ershoff and H. B. McWilliams. (Introduced by H. J. Deuel, Jr.)

From the Emory W. Thurston Laboratories, Los Angeles, California.

The bacteriostatic action of sulfaguanidine has been demonstrated by a number of investigators.[1-4] Inclusion of this drug in synthetic rations has resulted in reduction of growth,[2-6] prolongation of prothrombin time,[3,4] and various other symptoms[5,7,8] presumably due to inhibited synthesis of essential factors by intestinal microörganisms. These effects can be largely counteracted by liver extract,[3,4] *p*-aminobenzoic acid,[3,4,7] and inositol.[5] It is the purpose of the present communication to report the adequacy of synthetic rations containing both *p*-aminobenzoic acid and sulfaguanidine for reproduction in the albino rat.

Procedure and Results. The experimental diets consisted of two types: (1) those containing sulfaguanidine and (2) those with this drug omitted. These in turn were divided into rations with and without inositol. Four experimental diets were thus employed in addition to a natural food ration.

Synthetic B-complex factors were added to the experimental diets in the following quantities per kg of ration: 5 mg thiamine hydrochloride, 5 mg pyridoxine hydrochloride, 10 mg riboflavin, 100 mg nicotinic acid, 100 mg calcium pantothenate, 150 mg *p*-aminobenzoic acid, and 1 g choline chloride. When inositol was included it was added at a level of 400 mg per kg of ration. Daily supplements of 500 mg corn oil (Mazola), 0.25 mg alpha-tocopherol, and an A-D concentrate* equivalent to 50 U.S.P. units of vitamin A and 5 U.S.P. units of vitamin D were also administered.

TABLE I.
Experimental Diets Containing Synthetic B-Complex Supplements.

Ration Number	A	B	C	D
	g	g	g	g
Sulfaguanidine	0.5	0.5		
l(-) cystine	0.3	0.3	0.3	0.3
Salt Mixture*	4.5	4.5	4.5	4.5
Sucrose	72.7	72.7	73.2	73.2
Casein†	22.0	22.0	22.0	22.0
B Complex Supplement	X		X	
B Complex Supplement + Inositol		X		X
No. of animals in group	10	10	10	10

* Sure, Barnett, *J. Nutrition*, 1941, **22**, 499.
† Golden State Company, Ltd., San Francisco, California.

Fifty white female rats† weighing 30 to 35 g were weaned at 21 to 23 days of age and littermates divided among the 4 experimental groups listed above and a fifth maintained on a natural food ration supplemented once weekly with lettuce. Animals were kept in metal cages with screen bottoms to prevent access to feces; and sufficient food was administered to assure *ad lib.* feeding. Animals were weighed every fifth day, and vaginal smears were taken daily from the 60th day. At approximately 100 days of age, females were bred to males of proven fertility, and pregnancy dated from the finding of sperm in the vaginal tract.

Growth. No significant difference in rate

* Nopco Fish Oil Concentrate, assaying 800,000 U.S.P. units of vitamin A and 80,000 U.S.P. units of vitamin D per gram.

† From the animal colony, Department of Biochemistry, University of Southern California. Kindly provided by Dr. H. J. Deuel, Jr.

1 Marshall, E. K., Jr., Bratton, A. C., White, H. J., and Litchfield, J. T., Jr., *Bull. Johns Hopkins Hosp.*, 1940, **67**, 163.

2 Corwin, W. C., *Bull. Johns Hopkins Hosp.*, 1941, **69**, 39.

3 Black, S., McKibbin, J. M., and Elvehjem, C. A., Proc. Soc. Exp. Biol. and Med., 1941, **47**, 308.

4 Black, S., Overman, R. S., Elvehjem, C. A., and Link, K. P., *J. Biol. Chem.*, 1942, **145**, 137.

5 Martin, G. J., Proc. Soc. Exp. Biol. and Med., 1942, **51**, 56.

6 Light, R. F., Cracas, L. J., Olcott, C. T., and Frey, C. N., *J. Nutrition*, 1942, **24**, 427.

7 Mackenzie, J. B., Mackenzie, C. G., and McCollum, E. V., *Science*, 1941, **94**, 518.

8 Ashburn, L. L., Daft, F. S., Endicott, K. M., and Sebrell, W. H., *Pub. Health Rep.*, 1942, **57**, 1883.

of growth was observed between animals fed the experimental diets and those maintained on a natural food ration. Growth on diets containing sulfaguanidine was not inferior to that observed on similar diets with this drug omitted; nor was growth on inositol-deficient rations inferior to that observed on similar diets with inositol added. At 100 days of age averages of all groups fell within 200 ± 10 g.

Reproduction. No significant difference in reproductive performance was observed in any of the groups studied with the exception of that receiving both sulfaguanidine and inositol (diet B). Estrus cycles were regular throughout, and gestation and parturition were apparently normal. Young of normal birth weight were born in all cases, averaging 7 to 9 per litter, and with one exception all were born alive. In the group receiving both sulfaguanidine and inositol a marked reduction of fertility was observed. Of 9 animals bred on this ration (diet B) only four gave evidence of implantation; and of these only 2 cast litters. In litter mates receiving an identical ration with inositol omitted, or in litter mates receiving sulfaguanidine-free diets with or without inositol, the incidence of parturition following coitus approached 100%.

Under the conditions of this experiment dietary sources of inositol in excess of such quantities as may be present in casein were not required for reproduction of the female albino rat. Similarly, incorporation of sulfaguanidine to the extent of 0.5% in rations containing *p*-aminobenzoic acid was not incompatible with successful reproduction. Addition of inositol, however, to a purified ration containing sulfaguanidine resulted in marked reduction of fertility.

Lactation. Mothers failed to nurse their young in all cases on synthetic rations with the exception of those receiving a diet containing sulfaguanidine but free of inositol. Mothers failed to suckle whether the young were their own or foster young from mothers fed a natural food ration. Animals receiving sulfaguanidine but no inositol successfully nursed their young in a limited number of cases. Of 8 litters cast on diet A (sulfaguanidine—without inositol), 3 were successfully nursed, 10 of 18 young being carried through to weaning at a normal rate of growth. Of 21 remaining litters cast on experimental rations, the young died within 24 hours. Mothers on a natural food ration successfully nursed their young to weaning.

We are unable on the basis of present data to explain the above findings. It is not unlikely however that intestinal microörganisms play a part in the above results. Recent investigations have demonstrated that the intestinal flora of the rat synthesizes in varying degrees many of the B vitamins. Furthermore, it has been shown that alterations in the diet in respect to vitamins, carbohydrates, or other dietary substances may have a marked influence on the type and quantity of flora present. Martin,[9] for example, has made the interesting observation that although purified diets containing a synthetic B-complex supplement (thiamine, riboflavin, pyridoxine, choline chloride, nicotinic acid and calcium pantothenate) afford seemingly normal nutrition to the rat, administration of either *p*-aminobenzoic acid or inositol precipitated a deficiency that could be prevented by addition of the other factor. Under conditions of the present experiment we have been unable to confirm Martin's observation regarding the harmful effects of *p*-aminobenzoic acid administration; but his suggestion that inositol might stimulate growth of microörganisms which utilize and destroy some member of the B-complex, precipitating a deficiency of that factor, is interesting and may be pertinent to the above findings.

Summary. Incorporation of sulfaguanidine at a 0.5% level in purified rations containing *p*-aminobenzoic acid had no deleterious effects on reproduction in the female albino rat; similarly animals raised on inositol-free rations with or without sulfaguanidine were not inferior in growth, reproductive performance, or gross appearance to litter mates on identical rations with inositol added. Addition of inositol, however, to a purified ration containing sulfaguanidine resulted in marked reduction of fertility, which did not occur if either inositol or sulfaguanidine were omitted from the ration.

[9] Martin, G. J., *Am. J. Physiol.*, 1942, **136**, 124.

14381

Electron Microscope Study of Sperm.*

M. R. B. Baylor, A. Nalbandov, and G. L. Clark. (Introduced by L. V. Domm.)
From the Electron Optics Laboratories, Noyes Chemistry Laboratory, and the Department of Animal Husbandry, University of Illinois.

Originally the following study was undertaken with the hope of demonstrating degenerative changes occurring in the aged sperm which allow it to retain its fertilizing ability for a time but do not permit eggs fertilized by such sperm to develop into zygotes capable of hatching.[1]

Studies were begun with chicken sperm obtained by massage, and continued on bull sperm obtained by ejaculation into an artificial vagina. Attempts were also made to study human spermatozoa.

Human sperm, because of the presence of heavy secretions of the accessory glands which tend to coagulate and thus obscure the field of vision, were found in this preliminary study to be unsatisfactory.

All the bull sperm used came from fertile animals which are routinely used for artificial insemination. The bulls are permitted to ejaculate following a definite schedule and are not used to excess. This point is made in view of some of the findings reported below which do not agree with conclusions reached following sperm studies with the optical microscope.

The electron microscope preparations were made according to the directions given by Marton.[2] The bull and chicken semen were usually studied in a 1:100 dilution in distilled water. The suspension was applied to the collodion film with a bacteriological loop and allowed to dry at room temperature. Attempts were made to observe sperm in seminal fluid, however the density of the fluid obscured the field and made detailed study of the sperm impossible.

Since it is probable that drying of unfixed sperm may produce artifacts, part of the studies were undertaken on sperm fixed in alcohol, formalin, and mercuric chloride and some observations were made on unfixed sperm (hereafter referred to as "fresh sperm"). The stains used were: a silver preparation which is specific for lipoidal material and Harris' haematoxylin. In order to obtain stained sperm, semen was mixed with the water in a centrifuge tube, the mixture being centrifuged after each treatment and the residue containing the sperm cells being resuspended in the majority of cases in distilled water and occasionally in alcohol. The entire range of magnification of the electron microscope (1,000-100,000) was used in this study.

Results with Bull Sperm. The appearance of the head of bull sperm varied greatly de-

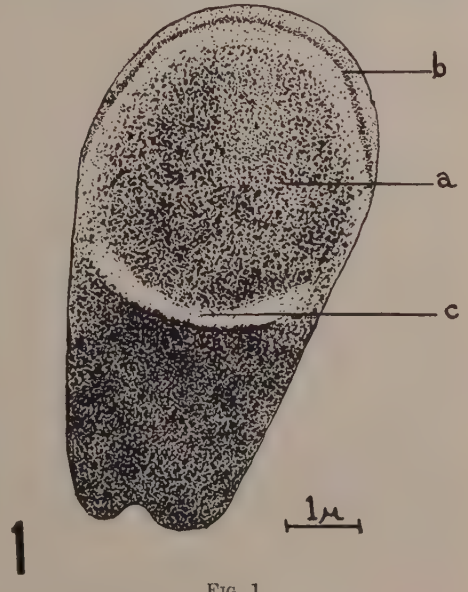
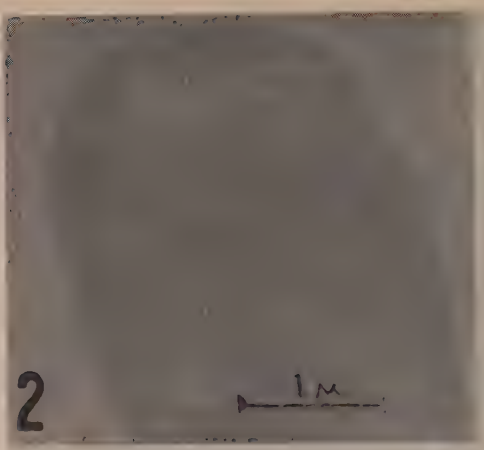


Fig. 1.

* We are indebted to Dr. Kruger, Veterinarian in charge of the Eastern Illinois Live Stock Improvement Co-operative, for supplying us with samples of bull semen and breeding records of these bulls.

[1] Nalbandov, A., and Card, L. E., *Poultry Sci.*, 1943, **22**, 218.

[2] Marton, *J. Bact.*, 1941, **41**, 397.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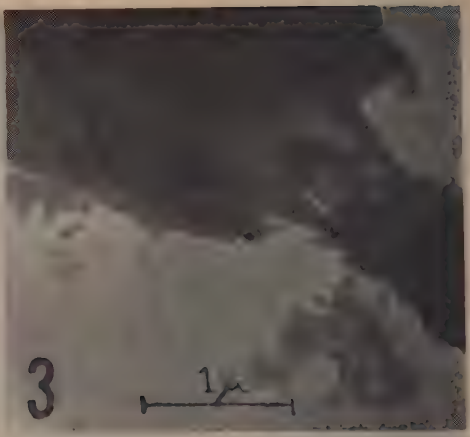
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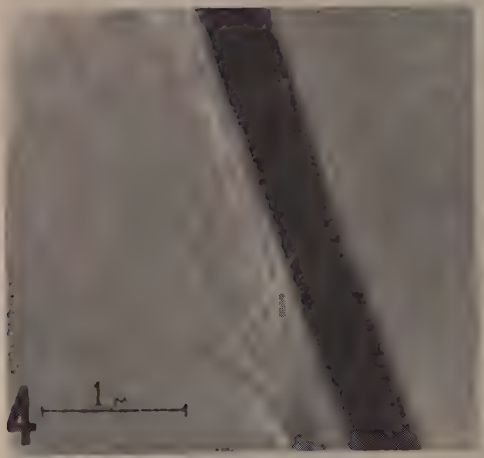
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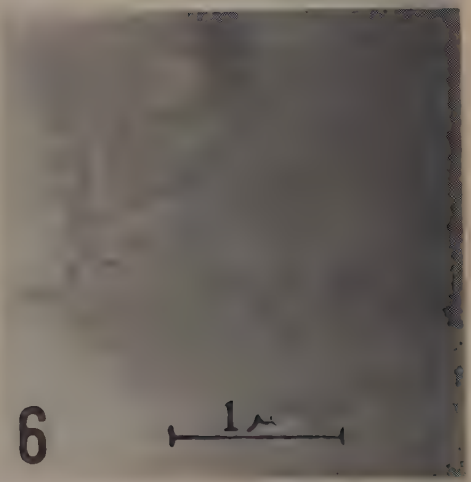
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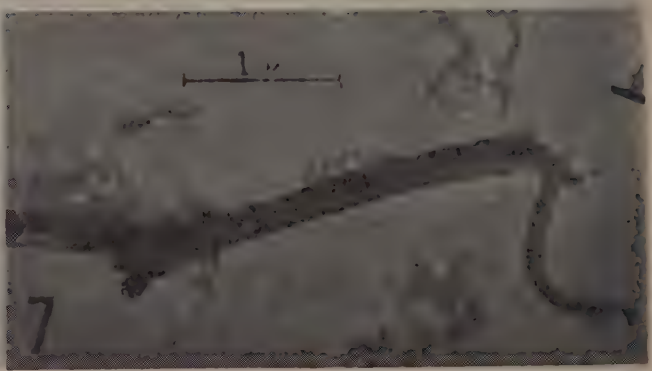
7
1μ

pending on whether the sperm was studied fresh, fixed, or stained. In all cases where no fixatives or stains were used, the head was enclosed in a protoplasmic cap which was always present but which varied in size depending on the individual sperm. (Fig. 2). This condition has been previously observed under the light microscope but has been described as a sign of immaturity or abnormality of the sperm.[3] In our study in which sperm samples from many bulls were used the protoplasmic cap appeared in all fresh specimens indicating that it is probably a normal part of the sperm. Presumably this protoplasmic cap is frequently dissolved when fixatives or stains containing solvents are used in preparing the sperm for examination under the optical microscope. We have found that when either stains or fixatives were employed, the cap was absent, or if present, was greatly shrunken and distorted.

In stained preparations the head appears to have 2 distinct zones of density. (Fig. 1.) These zones have been seen under the light microscope, but the electron microscope has revealed additional details. In some preparations the head appears to consist of 2 distinct and well-defined regions, one of which extends throughout the entire anterior portion of the head and seems to terminate in the posterior third of the sperm head. (Fig. 1 a.) The reasons for the difference in density within the sperm head may be twofold. The head may be flattened anteriorly thus offering less resistance to the electron beam which must penetrate it. Thus the anterior portion of the head would appear less dense than the region caudal to it which may be thought to be spherical in shape. Another explanation may be that the ovoid body occupying the anterior part of the head may consist of material less dense than the caudal region of the head. Completely surrounding this structure (nucleus?) is a narrow zone of even lighter density which stands out very clearly in stained preparations under the electron microscope but which is difficult to reproduce photographically. (Fig. 1 c.) Outside these regions of different densities the internal structure of the head appears to be homogeneous and no stains used up to now have shown any structures or regions of density which might suggest accumulation of chromosomes in either diffuse or condensed state.

In no case, even when the sperm was stained with silver to demonstrate lipoidal materials, was a structure observed that could be recognized as the acrosome. Correlating with optical microscope studies, the dense band seen in the lower half of the sperm head (Fig. 1) may be interpreted to delimit the acrosome, however, a more convincing electron microscope picture is needed.

The protoplasmic cap seems to envelop the whole head and in the lower half of the head it clings to the head outline rather closely.

The tail is connected with the head by means of 2 or more threadlike structures which become apparent when the tail breaks away from the head. (Fig. 3.) This condition frequently appears after centrifuging and staining.

The middle piece is uniformly dense and no mitochondria were demonstrable. The constriction between the middle piece and the end piece is easily seen and is essentially as observed under the light microscope.

The end piece (or main piece) in the unstained specimen appears hollow (Fig. 4) which may be due to the fact that the electron beam penetrates the central structure more easily than the thicker peripheral layer of cytoplasm surrounding it. In the stained tail in which the density of the central fibers has been increased over that of the enveloping cytoplasm, a very clear-cut axial filament is observed. This is very apparent in silver preparation. (Fig. 5.) In regions in which the cytoplasmic sheet has been broken one can see the naked filament exposed. (Fig. 5.)

The extremity of the tail (naked filament) in many preparations of fresh sperm appears brush-like in nature (Fig. 4) which suggests that the axial filament may be composed of a trunk of very thin but long fibers lying side by side and becoming exposed where the cytoplasm terminates. This is further substantiated by photographs of regions in which the end piece has broken, allowing the axial filament to flare out in a brush. (Fig. 6.) It is possible that the brushes observed at the

[3] McKenzie, F. F., Miller, J. C., and Bauguess, L. C., *Mo. Agr. Exp. Sta. Res. Bul.* 279, 1938.

extremity of the tail are due to such flaring out of the filament exposed by the breaking of the end piece, although in some cases such a break must have occurred very near the tip of the tail.

The fact that the filament and the extreme tip of the tail are composed of individual fibrils has been observed as early as 1886 and pictured by Ballowitz.[4] Since then this information has been disregarded by workers in the field of sperm morphology. Ballowitz calls these fibers the "Elementar-fibrillen" and ascribes to their presence the ability of sperm to move. He states, however, that in both birds and mammals the number of fibrils varies between 2 and 4. The electron microscope reveals that there are actually many more.

In stained preparations this brush phenomenon is never observed, possibly because the stain encases the fibers, cementing them into a rigid cylinder, thus not permitting them to flare out. In such preparations the naked terminal piece is clearly seen to be continuous with the axial filament of the end piece. (Fig. 7.) Usually following staining the very tip end of the tail appears clearly tapered although not brushed.

In the chicken sperm the head appears very dense, an acrosome is seen and the tail ends in a mass of long delicate fibers of sub-light-microscope dimensions. In chicken sperm also the middle piece breaks easily and releases a mass of fibers. This suggests that in the cock as well as in the bull the axial filament is made up of a bundle of many fibers.

We could not confirm the observation of Seymour and Benmosche[5] that the sperm head (human) has a suction disc nor could we find any "joints" in its middle pieces of any of the sperm types studied.

Summary. Fresh, unstained and unfixed samples of sperm from many fertile bulls studied under the electron microscope have shown that the anterior portion of the sperm head is always enveloped by a protoplasmic cap which appears damaged or disappears altogether if sperm are stained or fixed. This suggests that, contrary to results obtained with the optical microscope, the protoplasmic cap is not a sign of immature or abnormal sperm but is typical of normal sperm when these are examined without being exposed to solvents usually present in stains.

The tails end in a brush consisting of many free and very long filaments. Breaks in the main or end pieces of the tail have also shown flared brushes which make it seem likely that the axial filament consists of a bundle of fine fibers rather than a single relatively thick thread.

[4] Ballowitz, E., *Arch. f. Mikr. Anat.*, 1888, **32**, 401.

[5] Seymour, F. I., and Benmosche, M., *J. A. M. A.*, 1941, **116**, 2489.

14382

Utilization of Asparagus Juice in Microbiological Culture Media.

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This laboratory has a series of investigations in progress which relate to the possible usefulness of waste vegetable juices as microbiological culture media. The observation that asparagus-butt juice undergoes extraordinarily rapid microbiological spoilage, together with the interest now being shown in the production of antibacterial agents for use in the treatment of certain disease and wound infections, has led us to investigate the possibility of utilizing this juice as a medium for culturing some of the organisms that produce

antibacterial substances. Attention has also been given to the production of bacterial proteolytic enzymes.

The annual accumulation of asparagus waste (butt trimmings and culls) at packing houses and canneries in the United States has been estimated to range from 50,000 to 100,000 tons. This waste constitutes a major waste-disposal problem at asparagus processing centers. Marsh and Cruess[1] have previously recommended use of asparagus-butt juice to replace the brine solution used in canning asparagus.

Asparagus-butt juice appears to provide an excellent balance of nitrogen, sugar, and inorganic constituents for microbiological nutrition, though information concerning its precise chemical composition is rather meager. Analyses of various lots of juice prepared in this laboratory indicate that it contains approximately 4 to 5% solids, of which the major part is reducing sugar. The juice as pressed from fresh asparagus butts and completely clarified contains generally 0.07 to 0.13% total nitrogen.

Tyrothricin production. Asparagus-butt juice has been found to be suitable for the growth of *Bacillus brevis* and for the production of tyrothricin. Butt trimmings from both green and white asparagus that had been stored at 0°F for approximately 6 months were used as a source of juice. A cold-pressed juice was prepared by grinding the frozen butts in a meat grinder, followed by thawing and pressing in a hydraulic cider press. A hot-pressed juice was prepared by allowing the butts to thaw and then steaming 20 minutes at 100°C in a vegetable blancher. The steamed material was also pressed in a hydraulic cider press.

The pressed juices were clarified by filtration through a Mandler filter of medium porosity or through paper in a Buchner funnel after addition of diatomaceous filter aid. It was necessary to heat the cold-pressed juice several minutes at 100°C before filtration to precipitate heat-coagulable material and thereby to insure complete clarity after steam sterilization. The clarified juices were distributed in 50-ml portions in 250-ml Erlenmeyer flasks and sterilized by autoclaving for 20 minutes at 10 pounds of steam pressure. After sterilization the amount of normal sodium hydroxide required to adjust the pH to 7.5 was added to each flask aseptically.

The culture of *Bacillus brevis* used was the B. G. strain obtained from Dr. René Dubos at the Rockefeller Institute for Medical Research, New York City. The inoculum was prepared by making a transfer from a stock culture of beef agar to a beef agar slant and incubating 24 hours at 35°C. Transfer was then made with sterile water to a 25-ml portion of asparagus-butt juice which was then incubated 24 hours at 35°C. The flasks containing the 50-ml portions of juice media were then inoculated with 0.5 ml each of the inoculum and incubated for 2, 4, 6, and 10-day intervals at 35°C. Flasks were harvested in triplicate for tyrothricin assay after each of the indicated incubation periods.

The tyrothricin contents of the cultures were determined by a hemolytic method recently developed in this laboratory.[2] A standard preparation of tyrothricin was used as a control. The fractionation procedure of Dubos and Hotchkiss[3] with slight modification was used to determine actual recoverable yields of tyrothricin. The results obtained by the hemolytic method are shown in Table I.

A maximum of 2.0 g of tyrothricin per liter of culture medium was indicated in the hot-pressed green asparagus-butt juice after 10 days' incubation. This value was not checked by actual tyrothricin recovery. However, experience gained subsequently indicates that in most cases 90% or more of the tyrothricin found in asparagus juice cultures by the hemolytic assay method may be recovered by isolation.

Dubos and Hotchkiss[3] reported that yields on tryptone media may vary considerably, ranging from 0.02 to 0.50 g of tyrothricin per liter of culture medium. Stokes and Wood-

[1] Marsh, G. L., and Cruess, W. V., *Fruit Prod. J. and Am. Vinegar Industry*, 1942, **21**, 333.

[2] Dimick, Keene, *J. Biol. Chem.*, 1943, **149**, 387.

[3] Dubos, René J., and Hotchkiss, Rollin D., *J. Exp. Med.*, 1941, **73**, 629.

TABLE I.
Yields of Tyrothricin Obtained from Cultures of *Bacillus brevis* Grown in Asparagus Butt Juice Media.

Source of juice	Preparation of juice	Yields of tyrothricin by hemolytic assay.* Length of time of incubation			
		2 days g/l	4 days g/l	6 days g/l	10 days g/l
White asparagus	Cold pressed	0.48	0.57	0.95	1.23
" "	Hot "	0.47	1.00	1.27	1.68
Green "	Cold "	0.56	1.23	1.39	1.63
" "	Hot "	0.62	1.31	1.67	2.01

* Hemolytic assays by Dr. Keene Dimick. Each value is an average of 3 determinations.

ward[4] reported yields ranging from 100 mg to a maximum of 300 mg per liter in a variety of media under submerged conditions of culturing.

Antibacterial substance produced by Bacillus subtilis. *Bacillus subtilis* was observed to form an usually heavy pellicle after 24 hours' incubation when grown on asparagus-butt juice. The strain used was No. 231 of the culture collection of N. R. Smith of the Bureau of Plant Industry, Soils, and Agricultural Engineering, U. S. Department of Agriculture, Washington, D.C. While this study was in progress, Katznelson[5] reported that a substance toxic to microörganisms was produced by this organism.

A hot-pressed juice previously concentrated under vacuum at low temperature (25 to 30°C) to 9.4% soluble solids content was used as a medium to investigate antibacterial activity of *B. subtilis*. The juice was sterilized by Seitz filtration, distributed in 50-ml portions in sterile 250-ml Erlenmeyer flasks and the pH was adjusted to 7.0 with sterile normal sodium hydroxide. A suspension of *B. subtilis* in 10 ml of water washed from a beef agar slant after 24 hours' incubation at 30°C was used for inoculation.

The cultures were incubated at 35°C for 24 hours and harvested in triplicate. The pH of the harvested culture liquors was adjusted to 2.5 with hydrochloric acid, followed by sterilization at 10 lb steam pressure for 10 minutes. The 3 replicates were combined and the antibacterial activity determined by means of a serial dilution method against a number of test organisms including *Micrococcus conglomeratus* (Merck's MY strain), *Staphylococcus aureus* (U. S. Food and Drug Administration Strain No. 209), *Lactobacillus casei* (ATCC No. 7469), and three plant pathogens, *Erwinia amylovora*, *Phytomonas juglandis*, and *Phytomonas michiganensis*, obtained from Dr. P. A. Ark of the University of California, Berkeley, California.

The test medium used for determining antibacterial activity against all of the test organisms except *S. aureus* was a yeast-extract glucose broth. Beef broth was used in the case of *S. aureus*. The tubes containing the serial dilutions were incubated 24 hours at 30°C for all test organisms except for *S. aureus*, in which case the temperature was 35°C. A solution of tyrothricin was used as a standard in appropriate dilutions against the same test organisms. The results are shown in Table II.

Growth was measured by recording the difference in turbidity readings between the inoculated tubes and the uninoculated controls. A Klett-Summerson colorimeter was used for this purpose.

The data indicate the comparatively high inhibiting action of the *B. subtilis* cultures against all of the test organisms except the two Gram-negative plant pathogens, *E. amlyovora* and *P. juglandis*. The exceptionally high activity against *Phytomonas michiganensis* seems noteworthy. A gradual adaptation of this plant pathogen to the action of the antibiotic substance appears to take place, though not to the same degree as with *S.*

[4] Stokes, J. L., and Woodward, C. R., Jr., a paper presented at the joint meeting of the New Jersey and New York sections of the Society of American Bacteriologists in New York City, December 29, 1942.

[5] Katznelson, H., *Canadian J. Res.*, 1942, **20**, 169.

TABLE II.
Comparison of the Antibacterial Activity of a *Bacillus subtilis* Culture with That of a Tyrothricin Standard Against Various Test Organisms.

Test organisms	Growth of test organisms and dilution ratios	
	B. subtilis culture	Tyrothricin standard (0.5 g per liter)
Micrococcus conglomeratus	—1:10,240; 90%* at 1:20,480	—1:2,560; 90% at 1:5,120
Staphylococcus aureus	—1:640; 60% at 1:1,280	—1:80; 40% at 1:160
Lactobacillus casei	—1:40,960; 50% at 1:81,920	—1:640; 80% at 1:1,280
Erwinia amylovora	—1:80; 40% at 1:160	—1:20; 60% at 1:40
Phytomonas juglandis	—1:80; 15% at 1:160	—1:20; 50% at 1:40
Phytomonas michiganensis	—$1:10^{15}$†	—1:640; 40% at 1:1,280

* Percentages indicate estimates of growth based on turbidity readings. Negative signs indicate highest dilution ratios at which growth of the test organism was completely inhibited.
† Highest dilution tested; growth obtained at lower dilutions after longer incubation (See text).

aureus. Complete inhibition of *P. michiganensis* was obtained at a dilution of $1:10^{12}$ after 4 days' incubation, which was further reduced to the order of 1:5,000,000 after 6 days.

Antibacterial substances produced by fungi. Preliminary investigations of the suitability of media prepared from asparagus-butt juice indicated that both *Penicillium notatum* and *Penicillium citrinum* can be grown successfully with the production of good antibacterial activity in the culture liquors. Yields of citrinin in excess of 2 g per liter of culture medium can readily be obtained after 10 days' incubation at 30°C. These results compare favorably with those obtained by Raistrick and Smith[6] using a synthetic medium. Yields of penicillin have not been determined.

Proteinase production. Asparagus-butt juice has also been found to be suitable for the production of proteolytic enzymes, particularly by the *scaber* strain of *Bacillus subtilis* obtained from Dubos. Only minor supplementary treatment of the juice appears to be necessary for obtaining satisfactory yields. The proteinase obtained is similar to commercially produced bacterial proteolytic enzymes, and it is probable that asparagus concentrates can be used to replace more expensive industrial media.

Summary. The suitability of press juices prepared from waste asparagus butt trimmings as a source of the major components of media for culturing certain microörganisms of potential commercial importance has been indicated. The use of such media for the production of certain antibacterial substances and proteolytic enzymes appears to have particular promise.

[6] Raistrick, Harold, and Smith, George, *Chemistry and Industry*, 1941, **60**, 828.

14383

Effect of Inositol upon Rat Alopecia.*

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Introduction. Woolley[1] showed that inositol was needed to prevent alopecia in the mouse. Inositol has been reported to give a growth response in the rat by Pavcek and Baum[2] and in the chick by Hegsted *et al.*[3] Engel[4] has shown that inositol in addition to choline was needed by the rat in preventing fatty livers. Climenko and McChesney[5] have reported that inositol was needed for lactation in the rat while Sure[6] has shown that the addition of inositol to a purified diet was detrimental to lactation performance in the rat.

Welch and Wright[7] and Nielsen and Elvehjem[8] have shown that folic acid was needed by the rat for growth when it was fed with some of the sulfonamides.

The above studies were made with rats and mice fed purified diets. While investigating the deficiencies of a natural ration for reproduction in the pig and the rat[9,10] a hair loss was observed in the rat (Fig. 1) which could be cured or prevented by the addition of inositol to the ration.

Fig. 1.
Rat No. 38. Hair loss after 8 weeks on experiment.

Experimental. Pregnant females of Sprague-Dawley breeding were obtained 3-5 days before term. They were put on a basal ration composed of ground yellow corn 75.35, soybean oil meal 17.50, alfalfa meal 5.00, $CaHPO_4$ 1.00, NaCl (iodized) .50, $MnSO_4$ .01, haliver oil 2 drops weekly, and allowed to litter. Three days after the young were born the mother and litter were placed on their experimental rations as indicated below and growth records obtained. The young were weaned when they reached an average weight of 40 g.

Lot 1—Basal only.
Lot 2—Basal + folic acid preparation.
Lot 3—Basal + folic acid preparation + vit. B_6.
Lot 4—Basal + folic acid preparation + vit. B_6 + inositol.
Lot 5—Basal + inositol + vit. B_6.

The folic acid preparation used in these experiments was made according to the method of Hutchings, Bohonos, and Peterson.[11]

* Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. This work was supported in part by a grant from the Wisconsin Alumni Research Foundation.

1 Woolley, D. W., *J. Biol. Chem.*, 1941, **139**, 29.

2 Pavcek, P. L., and Baum, H. M., *Science*, 1941, **93**, 502.

3 Hegsted, D. M., Briggs, G. M., Mills, R. C., Elvehjem, C. A., and Hart, E. B., *Proc. Soc. Exp. Biol. and Med.*, 1941, **47**, 376.

4 Engel, R. W., *J. Nutr.*, 1942, **24**, 175.

5 Clemenko, D. R., and McChesney, E. W., *Proc. Soc. Exp. Biol. and Med.*, 1942, **51**, 157.

6 Sure, B., *J. Nutr.*, 1943, **26**, 275.

7 Welch, A. D., and Wright, L. D., *J. Nutr.*, 1943, **25**, 555.

8 Nielsen, E., and Elvehjem, C. A., *J. Biol. Chem.*, 1942, **145**, 713.

9 Ross, O. B., Phillips, P. H., and Bohstedt, G., *J. An. Sci.*, 1942, **1**, 86.

10 Ross, O. B., Phillips, P. H., and Bohstedt, G., *J. An. Sci.*, 1942, **1**, 353.

11 Hutchings, B. L., Bohonos, N., and Peterson, W. H., *J. Biol. Chem.*, 1941, **141**, 521.

TABLE I.
Growth Performance of Young.

Lot	No. of animals	Ration fed	Avg starting wt	Avg wt at end of 6 wks
1	6	Basal only	36	136
2	3	" + folic acid preparation	44	142
3	5	" + " " " + vit. B_6	38	106
4	5	" + " " " + " " + inositol	38	158
5	4	" + inositol + vit. B_6	40	116

This preparation was added to all the rations in an amount equivalent to 2% of solubilized liver extract. Inositol was fed at .3% of the ration and 60 γ of vitamin B_6 were given *per os* daily to each female. Vitamin B_6 was fed at the rate of 20γ per rat per day for the growth period.

Table I shows the growth performance obtained with these rats. The addition of the folic acid preparation and vitamin B_6 to the basal ration apparently threw the ration out of balance since a loss of hair was observed on the rats in lot III. Since there was no hair loss in the rats in lot IV where inositol was added, this dietary factor was given the rats of lot III to determine what the effect would be on the resumption of hair growth. The data in Table II show that inositol therapy was effective in curing hair loss under these conditions.

TABLE II.
Data on Hair Loss in Lot III.

Rat No.	Days before hair loss appeared	Days before inositol was added	No. of days required for complete furring
35	21	35	27
36	21	35	27
37	21	35	27
34	21	49	23
38	21	56	35

The type of hair loss is shown in Fig. 1. The hair loss starts in the dorsal part of the head and proceeds bilaterally along the sides to the tail region and then downward to the hind legs. With inositol therapy the hair returns inversely to the loss, proceeding from the caudal portions forward (Fig. 2).

In Table I the data for lots III and IV also show that inositol gives a growth response in addition to preventing loss of hair. The results indicate that the folic acid preparation likewise gives a growth response but in order to do so it must have added amounts of inositol and vitamin B_6. These data on growth show that the basal ration was deficient in several factors. The addition of certain factors without certain others seemed to give an imbalance which was detrimental to growth. This may be due to an interaction of the vitamins, to some change in intestinal synthesis, or to other unknown causes.

FIG. 2.
Rat No. 38. 28 days on inositol therapy.

Discussion. Pavcek and Baum[2] were able to cure spectacled eye in the rat by the addition of inositol to their purified diet. Within 3 days after inositol therapy definite signs of hair restoration were evident. Their results agree with this study since hair growth was observed to begin after 2-3 days of inositol therapy.

It would appear that this type of rat alopecia is a strictly inositol deficiency. In these experiments a large amount of inositol was fed and no attempt to purify further or recrystallize the inositol was made and thus the possibility of contaminating substances was not completely eliminated. In view of Woolley's

results with the mouse[1] it would appear that repeated recrystallization of inositol does not materially alter the anti-alopecia properties of inositol.

Summary. In the rat fed a ration of naturally occurring feeds inositol supplementation gave a growth response and it also cured and prevented a widespread loss of hair. When a folic acid preparation, together with inositol and B_6 was fed a better growth rate was obtained than when the folic acid preparation was omitted.

These data clearly show that a natural ration composed of yellow corn, soybean oil meal, $CaHPO_4$, $CaCO_3$, $MnSO_4$, NaCl, and 5% alfalfa hay contains insufficient inositol, folic acid concentrate and B_6 for normal growth and proper hair and fur development in the rat. It would appear that the phytin present in this ration was either inadequate to supply the required inositol or that it was unavailable.

14384 P

Heredity and Distribution of the Rh Blood Types.*

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In a preceding paper[1] a theory of 6 allelic genes was proposed to account for the existence of variants within the Rh-positive type of human blood. The present communication is a preliminary report of investigations on the Rh blood types in families, and their distribution in different races, these studies having been carried out in order to test the 6-gene theory.

In Table I is presented, in simplified form, the 8 Rh blood types and their reactions with the 3 sorts of anti-Rh agglutinins, namely, anti-Rh (standard, and agglutinating the bloods of approximately 85% of white individuals), anti-Rh_1 (giving about 70% positive reactions with bloods of white individuals), and anti-Rh_2 (giving about 35% positive reactions). Among white individuals approximately 95% fall into one of the 4 types listed in the left half of the table, while the 4 types in the right half of the table together make up only 5% of the population. For analyzing data, it is sometimes convenient to disregard the reactions of the standard anti-Rh sera, and in that way the two halves of the table are fused, giving rise to 4 classes, comparable serologically and genetically to the 4 blood groups. Obviously, each class consists of 2 distinct Rh types.

In Table II are summarized our findings to date on the heredity of the Rh blood types in 94 families (83 white, 11 negro). It will be seen that among the 274 children there is only a single apparent exception to the theory. In this case (a negro family) the mother was Rh-negative, her husband Rh′, and the child Rh_2. However, it was also found that the supposed father belonged to type N and the child to type M, so that this was evidently an illegitimate child. Of particular interest are the 4 matings Rh-negative $\times$ Rh_1Rh_2 in which there were 8 Rh_1 and 8 Rh_2 children. These findings are entirely comparable to those obtained in the mating O $\times$ AB in studies on the heredity of the Landsteiner blood groups. In addition to the 94 complete families, tests were carried out on mother-child combinations, and among 73 mothers with 123 children, not once were the combinations Rh-negative mother with type Rh_1Rh_2 child or type Rh_1Rh_2 mother with Rh-negative child encountered. The findings will be discussed in greater detail in the full report[2] which will also contain the complete family

* Aided by a grant from the United Hospital Fund of New York City.

1 Wiener, A. S., PROC. SOC. EXP. BIOL. AND MED., in press.

2 Wiener, A. S., Sonn, E. B., and Belkin, R. B., in preparation.

TABLE I.
Classification of the Rh Blood Types.

Class	Testing sera: Anti-Rh_1	Anti-Rh_2	Anti-Rh	Type	Testing sera: Anti-Rh_1	Anti-Rh_2	Anti-Rh	Type
U	+	—	+	Rh_1	+	—	—	Rh′
V	—	+	+	Rh_2	—	+	—	Rh″
UV	+	+	+	Rh_1Rh_2	+	+	—	Rh′Rh″
W	—	—	—	Neg.	—	—	+	Rh

TABLE II.
Heredity of the Rh Blood Types in 94 Families.

Mating	No. of families	Children of types: Neg.	Rh_1	Rh_2	Rh_1Rh_2	Rh	Rh′	Totals
Neg. × Neg.	2	9	—	—	—	—	—	9
Neg. × Rh_1	17	13	36	—	—	3	—	52
Neg. × Rh_2	5	1	—	6	—	—	—	7
Neg. × Rh_1Rh_2	4	—	8	8	—	—	—	16
Neg. × Rh	1	3	—	—	—	1	—	4
Neg. × Rh′	1	—	—	(1)	—	—	—	1
Rh_1 × Rh_1	15	4	44	—	—	4	1	53
Rh_1 × Rh_2	15	3	13	7	13	—	1	37
Rh_1 × Rh_1Rh_2	19	—	27	6	22	—	—	55
Rh_2 × Rh_2	1	1	—	1	—	—	—	2
Rh_2 × Rh_1Rh_2	4	—	1	5	3	—	—	9
Rh_2 × Rh	2	—	—	1	—	4	—	5
Rh_1Rh_2 × Rh_1Rh_2	7	—	4	—	17	—	—	21
Rh′ × Rh_1Rh_2	1	—	1	1	1	—	—	3
Totals	94	34	134	36	56	12	2	274

TABLE III.
Racial Distribution of the Rh Blood Types.

Race	No. tested		Neg.	Rh_1	Rh_2	Rh_1Rh_2	Rh	Rh′	Rh″	Rh′Rh″
White	500	Approx. %	12.75	50	15.5	17	2.5	2	0.25	—*
Negroes‡	143†	No.	13	31	30	15	51	1	—	—
		%	9.1	21.9	21.0	10.5	35.7	0.7	—	—
Chinese§	80	No.	1	46	4	28	1	—	—	—
		%	1.25	57.5	5.0	35.0	1.25	—	—	—

* This theoretically possible type has not yet been encountered, which is not surprising considering the extremely low value for the expected frequency of the type.

† Two bloods gave atypical reactions.

‡ Wiener, A. S., Belkin, R. B., and Sonn, E. B., detailed paper in preparation.

§ Wiener, A. S., Sonn, E. B., and Yi, C. L., detailed paper in preparation.

data, including the blood groups, sub-groups, and M-N types.

Studies on the distribution of the Rh blood types among whites, negroes, and Chinese revealed very striking differences among these races. These findings are in sharp contrast to the results of the blood grouping and M-N tests for the same races, since, for example, the distributions of the M-N types among whites, negroes and Chinese do not differ greatly. However, we did observe that all the group A and AB individuals among the Chinese belong to sub-group A_1, so that sub-group A_2 is apparently lacking in this race.

In Table III we have tabulated our findings on the distribution of the Rh blood types among negroes and Chinese as compared with their approximate distribution among white individuals (the latter being based on a series of more than 500 persons). It is of interest to note the very high incidence of the type Rh among negroes, in contrast to its low incidence in whites and Chinese. On the other hand, among Chinese the Rh-negative type is

practically lacking[3] while the frequencies of types Rh_1 and Rh_1Rh_2 are high. In fact, if the single Rh-negative individual and the single individual of type Rh are discounted as possibly due to racial admixture, then the distribution in our series of Chinese reduces itself to a two-factor scheme, quite similar, for example, to the distribution of the M-N types among American Indians.

The application of simplified gene frequency formulæ calculated from the frequencies of classes U, V, UV, and W supplies further support for the hypothesis of multiple allelic genes. However, the family and statistical data are still inadequate to test the theory satisfactorily with regard to the rarer types, Rh′, Rh″, and Rh′Rh″.

[3] Levine and Wong found only 0.7% Rh-negative individuals among 150 Chinese (*Am. J. Obst. and Gyn.*, 1943, **45**, 832).

14385

Comparison of Dextran Synthesis by Leuconostoc Enzyme with Starch Synthesis by Potato Phosphorylase.*

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Earlier papers[1,2] described the synthesis from sucrose of a serologically reactive dextran by cell-free enzymes obtained from a species of streptococcus-like bacteria, *Leuconostoc mesenteroides*. Some evidence was obtained which indicated that the course of the synthesis follows the equation:

$$n \text{ sucrose} \rightarrow \underset{\text{dextran}}{(\text{glucose anhydride})_n} + n \text{ fructose} \quad (1)$$

However, that mode of dextran formation was not proved and, indeed, analogy with the enzymatic syntheses of glycogen and starch would suggest the possibility that glucose-l-phosphate might participate as an intermediate substance in the conversion of sucrose to dextran. That possibility was made more significant by a recent report[3] that suspensions of *Leuconostoc mesenteroides* can phosphorolyse sucrose with the production of glucose-l-phosphate. If glucose-l-phosphate does serve as an intermediate substance in the formation of dextran, the synthesis of that polysaccharide by the bacterial enzyme would fall in the same class of reactions as the syntheses of glycogen and starch by the phosphorylases of other biological origin.

In order to get experimental data on this question, a comparison was made between leuconostoc enzyme and potato phosphorylase in respect to their action upon sucrose and upon glucose-l-phosphate. The leuconostoc enzyme was obtained from sucrose broth cultures of the strain used in previous studies; it was prepared by an improved method which will be described in another paper, and was of much greater potency than any of the preparations utilized in the earlier studies. The potato phosphorylase was prepared according to the first steps in the procedure described by Green and Stumpf.[4]

A set of 3 test mixtures was prepared with each enzyme. The first consisted of equal parts of enzyme solution and of 0.2 M sucrose in 0.1 M acetate buffer, pH 5.6; the second

* This study was supported by a grant from the Ruth B. Ettinger Fund. We are indebted also to Dr. Walther F. Goebel of the Hospital of the Rockefeller Institute for Medical Research who furnished the glucose-l-phosphate used in the experiments.

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[2] Hehre, E. J., and Sugg, J. Y., *J. Exp. Med.*, 1942, **75**, 339.

[3] Kagan, B. O., Latker, S. N., and Zfasman, E. M., *Biokhimiya*, 1942, **7**, 93.

[4] Green, D. E., and Stumpf, P. K., *J. Biol. Chem.*, 1942, **142**, 355.

TABLE I.
Comparison of Leuconostoc Extract and Potato Phosphorylase in Respect to Action upon Sucrose and upon Glucose-1-phosphate.

Source of enzyme	Substrate	Properties of test mixtures incubated 4 hours at 25°C: Appearance	Precipitate with 1.25 vol. alcohol	Serological reactivity	Color with iodine	Reducing sugar mg/ml	Inorganic phosphate mg/ml
Leuconostoc	sucrose	opalescent	++++	20,000	yellow	6.2	<0.001*
	glucose-1-phosphate	clear	0	40	"	0.1	0.03
	buffer control	"	0	40	"	0.1	<0.001*
Potato	sucrose	"	±	0	"	0.3	0.07
	glucose-1-phosphate	cloudy	++++	0	blue-black	0.3	6.4
	buffer control	clear	±	0	yellow	0.3	0.07

* No phosphate was detected by the Fiske-Subbarow[6] procedure or by the Deniges[7] method, either before or after the mixture had been digested with sulfuric and nitric acids.

comprised equal parts of enzyme and of 0.2 M glucose-1-phosphate in 0.1 M acetate buffer, pH 5.6; and the third or control mixture consisted of equal parts of enzyme and of the buffer solution. All the test mixtures were incubated for 4 hours at 25°C and were then examined for opalescence or cloudiness and for amount of precipitate formed upon addition of 1.25 volumes of alcohol; each mixture was also tested for reactivity with type 2 antipneumococcus serum, color with iodine, reducing sugars[5] and inorganic phosphate.[6] The data are summarized in Table I.

Results. It is evident (Table I) that the leuconostoc enzyme, when incubated with sucrose, caused the formation of abundant amounts of dextran together with the liberation of free reducing sugar (fructose); and that the potato phosphorylase, when incubated with glucose-1-phosphate, caused the formation of abundant amounts of starch together with the liberation of inorganic phosphate. In contrast, the leuconostoc enzyme had no demonstrable action upon glucose-1-phosphate and the potato phosphorylase had none upon sucrose. The lack of reactivity with glucose-1-phosphate on the part of the leuconostoc enzyme which was so reactive with sucrose is good evidence that glucose-1-phosphate is not an intermediate substrate in the enzymatic synthesis of dextran from sucrose. The absence of any appreciable amount of phosphate in the system which formed abundant dextran suggests that the enzymatic synthesis of that polysaccharide from sucrose does not require the mediation of any phosphorylated sugar substrate, but occurs by a direct condensation of the sucrose in the way indicated by Equation 1.

Though the enzymes operate upon different substrates and yield chemically different products, the synthesis of dextran by leuconostoc enzyme does have a fundamental point of similarity to the syntheses of starch and glycogen by the various phosphorylases. That is, in each instance the substrate (sucrose or glucose-1-phosphate) contains the basic unit of the final polymer product in the form of a glycoside radical that is exceedingly easily split off by acids. The same relationship obtains in the enzymatic synthesis of levan, because here also the basic unit (fructose anhydride) is contained as a glycoside radical in the substrates (sucrose and raffinose) from which the polymer product is derived.[8-10]

Summary. Comparison with starch synthesis by potato phosphorylase indicates that dextran synthesis from sucrose by leuconostoc enzyme does not require the mediation of any phosphorylated sugar.

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[6] Fiske, C. H., and Subbarow, Y., *J. Biol. Chem.*, 1925, **66**, 375.

[7] Deniges, G., *C. R. Acad. Sc.*, 1927, **185**, 777; 1928, **186**, 318.

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[9] Harrison, F. C., Tarr, H. L. A., and Hibbert, H., *Canad. J. Research*, 1930, **3**, 449.

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14386 P

Effect of Cholecystectomy on Liver Function.

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The cholecystectomized animal or man remains in apparently good health over a long period of time. However, closer study has shown that the removal of the gallbladder may result in dilatation of the biliary tree and hepatitis (*cf.*[1]). Cantarow, Gartman, and Ricchiuti[2] reported that cholecystectomy in man sometimes temporarily impaired the hepatic excretion of bromsulphalein when tested 24 hours after cholecystectomy. Bergh, Sandblom, and Ivy[1] found no change in the liver function of cholecystectomized dogs at intervals of from one week to 15 months after operation; they used the bromsulphalein test (2 mg dose), Van den Bergh, the icteric index as criteria. Since then the 5 mg dose of bromsulphalein has been shown to be more sensitive than the 2 mg dose in detecting hepatic damage. For detecting hepatic damage produced by CCl_4 in dogs[3] the change in serum phosphatase has been shown to be nearly as sensitive as the bromsulphalein test (5 mg dose). Therefore, the effect of cholecystectomy on liver function was studied in dogs by means of the bromsulphalein test (5 mg dose) and serum phosphatase test.

Methods. Serum phosphatase was determined according to the method of Bodansky.[4] Six units of phosphatase per 100 cc of serum was the upper limit of normal in control determinations. Five mg of bromsulphalein per kilo of body weight were injected intravenously and dye retention was determined in the serum one-half hour later, as described in earlier experiments.[5] A blue-glass filter was used in comparing the concentration of dye. Normal dogs vary from 2 to 12% retention of dye at the end of one-half hour, the upper limit of normal being taken as 15%.

Twelve cholecystectomized dogs, operated under ether anesthesia, were studied for 24 to 70 days post-operatively. Five of the dogs were fed the stock laboratory diet of meat scraps, bread, bones, cod liver oil, and yeast. Seven dogs were fed a modified form of Cowgill's casein diet,[5] plus a daily supplement of yeast.

Results. Ten of the dogs showed a definite rise in serum phosphatase, values of 8 to 34 units per 100 cc of serum being found. One showed no change in serum phosphatase during 31 days of observation and a second only a transitory rise 43 days post-operatively, returning to normal after the 61st day. In only one of the 10 dogs did the serum phosphatase return to normal during the period of observation, even though studies were continued for 50 to 70 days in some of the dogs. (Table I.)

Of the 10 cholecystectomized dogs showing a rise in serum phosphatase only 4 dogs had consistent retention of bromsulphalein, the dye retention varying from 25 to 60% in these dogs. The dogs having an abnormal retention of bromsulphalein were not necessarily those having the highest serum phosphatase values.

That the increase in serum phosphatase is not merely a post-operative reaction is shown by the fact that 2 days after operation there was generally only a slight rise in phosphatase activity, the increase being greater 5 to 10 days post-operatively. Also, the increased serum phosphatase activity was maintained for as long as 70 days, the maximum period of observation.

Discussion. Following cholecystectomy the

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[2] Cantarow, A., Gartman, E., and Ricchiuti, G., *Arch. Surg.*, 1935, **30**, 865.

[3] Drill, V. A., and Ivy, A. C., *Fed. Proc.*, 1943, **2**, 10.

[4] Bodansky, A., *J. Biol. Chem.*, 1937, **120**, 167.

[5] Drill, V. A., and Hays, H. W., Proc. Soc. Exp. Biol. and Med., 1940, **43**, 450.

TABLE I.
Effect of Cholecystectomy on Serum Phosphatase and Bromsulphalein Retention.*

Dog No.	1		2		3		4		5	
Days post-operatively	Brom. reten.	Serum phos-phatase	Brom. reten.	Serum phos-phatase	Brom. reten.	Serum phos-phatase	Brom. reten.	Serum phos-phatase	Brom. reten.	Serum phos-phatase
Control	8	5.81	5	5.95	12	4.83	10	5.60	13	4.62
2	10	11.86	15	7.20	10	6.80	50	5.99	25	9.33
4-5	5	4.71	5	3.63	10	26.13	30	7.17	12	8.75
6-10	5	8.55	10	8.70	5	15.51	50	10.01	60	26.10
11-20	12	11.28	5	13.21	10	9.92	15	10.05	12	15.56
21-30	15	11.63	5	10.03	10	8.99	60	11.89	25	12.47
41-50	35	5.52	8	16.37	35	22.61				
51-70	12	10.25	10	9.87	10	12.09				

* More determinations were made at closer intervals than illustrated by these 5 dogs in the table.

biliary ducts are known to dilate and a slight hepatitis has also been observed. Cholecystectomy seems to cause incompetence of the sphincter of Oddi, thus allowing transmission of pressure into the ducts from the duodenum.[1,6,7] Bergh *et al.*[1] reported that 8 of 11 cholecystectomized dogs still had grossly dilated ducts 9½ to 15 months after operation. It is thus not surprising that the serum phosphatase of cholecystectomized dogs was still above normal 70 days following operation. Since only 4 of the 10 dogs showing a rise in serum phosphatase had an increased retention of bromsulphalein, serum phosphatase is a more sensitive index than the bromsulphalein test for the slight functional biliary obstruction that is responsible for dilatation of the bile ducts. The mechanism by which the increased intraductal pressure raises serum phosphatase is unknown. Studies have been reported on phosphatase excretion in the bile of cholecystectomized-bile fistula dogs.[8] In such dogs the rise in serum phosphatase could be correlated with either a decreased excretion of bile phosphatase, presumably leading to a higher blood level of the enzyme, or with an increased biliary secretion of phosphatase which was also associated with a blood level above normal.

Summary. Ten of 12 cholecystectomized dogs showed a definite rise in serum phosphatase following operation. In one of the 10 dogs the serum phosphatase value returned to normal, whereas in the other dogs the rise in serum phosphatase was maintained throughout the period of observation, the maximum period being 70 days. Of the 10 cholecystectomized showing a rise in serum phosphatase, only 4 developed an increased retention of bromsulphalein.

6 Ivy, A. C., *Physiol. Rev.*, 1934, **14**, 1.

7 Sandblom, P., Bergh, G. S., and Ivy, A. C., *Ann. Surg.*, 1936, **104**, 702.

8 Drill, V. A., Annegers, J. H., Snapp, F. E., and Ivy, A. C., *Fed. Proc.*, 1943, **2**, 9.

14387 P

Active Hypersensitivity from Inhalation of Finely Atomized Fluid Antigens.*

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Recent reports indicate that influenza may be successfully treated by inhalation of finely atomized particles (aerosol) of specific antiserum.[1-3] If further investigation confirms these reports, it is likely that this form of treatment will be attempted on a large scale. Because the supply of human convalescent serum is limited, antiserums of other than human origin will in all probability be used. The possibility must be considered that a foreign serum used in this manner may produce active as well as passive sensitization and thus bring about a dangerous state of hypersensitivity. It is also of great importance to know whether or not this method of treatment will produce serious allergic reactions such as anaphylactic shock, asthma, or pneumonitis in patients who are already hypersensitive to the particular antiserum used.† In order to answer these questions as well as to explore further some of the basic mechanisms which concern "natural" acquired sensitivity, blood serum from animals commonly used for the production of therapeutic antiserums (rabbits, goats, swine, and horses) and egg albumin were tested in a manner which would simulate their use under the conditions mentioned above.

The procedures employed in this study were essentially the same for each of the 5 antigens tested as illustrated by the following example: A group of 24 guinea pigs weighing 300-350 g each was subdivided into 3 groups of 8 each. Group 1 was placed in a closed chamber and exposed for 20 minutes to an atmosphere into which undiluted sterile swine serum was continuously atomized. This administration of serum by inhalation was repeated daily for 5 successive days. The finely atomized particles of serum were diffusely and densely distributed throughout the 27x14x14-inch exposure chamber. Group 2 received a single intraperitoneal injection of 0.5 cc of swine serum. Group 3 received no treatment and served as controls.

Fourteen days after the first treatment with swine serum, precipitin titers were determined on the pooled serums from each group using the antibody dilution method of Cannon and Marshall; see Table I.

Four weeks after guinea pigs had been injected with or had first inhaled swine serum, animals of all 3 groups were exposed to finely atomized swine serum. In every instance, the animals which had been previously treated, either through inhalation or by injection of

TABLE I.
Antibody Response Following Administration of Antigen.

Antigen used	Manner of administration	Antibody titer
Swine Serum	Inhalation (Aerosol)	<1:10
	Injection	*
Rabbit Serum	Inhalation (Aerosol)	<1:10
	Inj.	1:512
Goat Serum	Inhalation (Aerosol)	1:40
	Inj.	1:640
Horse Serum	Inhalation (Aerosol)	1:80
	Inj.	1:80
Egg Albumin	Inhalation (Aerosol)	<1:10
	Inj.	1:10

* Serum was unusable because of bacterial contamination.

* This investigation was aided in part through the Influenza Commission, Board for the Investigation and Control of Influenza and Other Epidemic Diseases in the Army, Preventive Medicine Division, Office of the Surgeon General, U. S. Army.

1 Smorodintseff, A. A., Gulmow, A. G., and Tshalkina, O. M., *Z. f. Klin. Med.*, 1940, 756.

2 Henle, W., Stokes, J., Jr., and Shaw, D. R., *J. Immunol.*, 1941, **40**, 201.

3 Smorodintseff, A. A., and Shishkina, O. I., *Arkiv. Biologischeskikh Nauk*, 1938, **52**, 132.

† References to literature concerning immunologic response to inhaled antigens may be found in Ratner, B., *Allergy, Anaphylaxis and Immunotherapy*, Williams and Wilkins, Baltimore, 1943.

swine serum, exhibited varying degrees of anaphylactic shock. This was characterized by dyspnea, coughing, sneezing, roughening of the coat, and prostration. A moderate to marked hyperemia and edema of the conjunctivæ also developed. Swine, rabbit, goat, and horse serum each caused approximately the same degree and type of allergic reaction in specifically sensitized animals regardless of whether the initial, sensitizing dose had been inhaled or given parenterally. Of 56 sensitized animals which were treated in this manner, 3 died in the chamber during exposure to an atomized specific antigen. Two of these had been sensitized by a single intraperitoneal injection of 0.3% crystalline egg albumin, the third by inhalation of the same substance. Other than this, however, anaphylactic reactions were sub-lethal and symptoms usually subsided within a few hours after each exposure to aerosol. Control animals gave slight or no reactions. Four weeks after sensitization by inhalation of various antigens, 14 guinea pigs were injected intravenously with 0.3 to 0.5 cc of the specific antigen. Twelve of the 14 animals in this group died of typical anaphylactic shock.

It has been suggested that repeated inhalations of small doses of anti-influenzal serum might be used prophylactically. A second series of experiments, using goat serum alone, was conducted to determine whether or not widely spaced inhalations of smaller quantities of serum would also produce a state of active hypersensitivity. Guinea pigs and rabbits were exposed to aerosols of undiluted or diluted (1:10) goat serum for 20 minutes at weekly intervals. Upon the third of the weekly exposures, mild reactions were observed. By the fifth week of such treatment, allergic reactions were quite marked and were in every way similar to those previously described.

Histologic studies of tissues from animals which were autopsied two days following the last of several (5 or 6) 20-minute exposures to atomized antigen revealed acute inflammatory changes in lungs, trachea, and nasal mucosæ. These were present to some extent in all of the animals, including controls, but were more pronounced in those which had been previously sensitized by injection or inhalation of the specific antigen.

From these observations it is apparent that active sensitization can be readily accomplished by inhalation of finely atomized fluid antigen and that serious allergic reactions, even fatal anaphylactic shock, may occur when hypersensitive animals inhale aerosol of specific antigen.

14388

Experimental Basis for the Chemotherapy of *Trichomonas vaginalis* Infestations. I.

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Clinical reports clearly indicate that an entirely satisfactory therapeutic agent is not established for the treatment of *Trichomonas vaginalis* infestations. *In vitro* studies of the trichomonacidal effect of various compounds can supply only collateral data for future therapeutic trial. Such factors as prolonged exposure of the parasite to the medication mixture, the intervention of tissue exudates, and preparatory cleansing and drying of the site of infection lead to divergence of *in vitro* and clinical findings.

The data reported in this paper were obtained from tests of a number of proprietary compounds which were solicited from the manufacturers, as well as others selected for reasons of theory or convenience. The list will be enlarged in future studies by investigation of many compounds suggested by clinical literature and will be supplemented by

reports dealing with the inhibiting action of these test preparations.

The completion of this study was made possible by the generous interest and support of Doctor E. D. Plass and Doctor M. E. Barnes.

Method. Various dilutions of the test compounds were made up in sterile, cotton stoppered tubes to a final volume of 3.8 cc. A human serum content of 25% was established either by adding 1 cc of 100% serum to 2.8 cc of drug solution or by dissolving the drug directly to the final dilution in 25% serum. In the first method the drug solution was made up to a calculated concentration such that the addition of 1 cc of serum and 0.2 cc of inoculum would reduce it to the required final dilution. A useful formula for computing the dilutions is given in Appendix B. In either case the serum was previously adjusted to pH 6 with N/1 HCl. These solutions were prepared aseptically and throughout the test period they were maintained at 37°C in a constant temperature bath. The final volume of the test mixture was 4 cc and consisted of 0.2 cc of inoculum, plus 2.8 cc of drug solution and 1 cc of 100% serum, or 3.8 cc of 25% serum containing the drug in the required final dilution.

An inoculum of 400,000 organisms was introduced by adding 0.2 cc of a 48-hour

TABLE I.
Compounds Without Demonstrated Trichomonacidal Activity.

Chemical name	Synonym or trade name	Max. conc., one part in:	Max. exposure, min.	pH		Solvent
				Test mixture	Control	
7-iodo-8-hydroxyquinoline-5 sulfonic acid	Chiniofon Yatren	70	10	6.2	6.36	Water
5,7,-diiodo-8-hydroxyquinoline	Diodoquin	(70% sat.)*	120	5.5	5.62	"
		6% suspension	10			
Bile Salts		70	60	6.18	6.3	"
p-Carbamyl-aminophenyl-arsonic acid	Carbarsone Arsanilic acid	100	120	7.79	5.27	N/1 NaOH
Acetarsone + boric acid + CHO	Devegan	(70% sat.)*	60	5.3	5.47	Water
2,4-dihydroxy-n-hexylbenzene	Caprokol Hexylresorcinol	2800 (see Table II)	10	5.94	5.92	"
Triiodomethane	Iodoform	(70% sat.)*	15	6.5		"
Triacetylpyrogallol	Lenigallol	10% suspension	10	5.1	6.36	"
	Meta Cine	(70% sat.)*	15	3.11	5.78	"
		6% suspension	10			
Sodium perborate + hydroxyquinoline + aluminum silicate + sodium borate + boric acid	Pulvis alkalinus fungi	(70% sat.)	60	8.82	5.5	"
Quinine sulfate		1000	60	5.43	5.47	"
Sodium anthraquinone-sulfonate		140	60	6.1	6.3	"
Sodium 2,5-bisulfanil-amidobenzene-sulfonate		1000	15	5.6	5.38	"
Sodium N'-cinnamoyl-sulfanilamide		1000	15	5.8	5.38	"
Sulfadiazine		14000	15			"
Sulfaguanidine		750	60	5.5-6.0	5.47	"
Sulfamethyldiazine		4700	60	5.5	5.47	"
Sulfanilamide		178	180	5.8	5.58	"
Sulfapyridine		3500	60	5.4	5.47	"
Sulfathiazole		1100	60	5.57	5.6	"
Sulfathiazole + *B. lactose*		(70% sat.)*	60	5.69	5.71	"
Alkyl dimethyl-benzylammonium chlorides	Zephiran	1400	15	6.2		"

* Sat.—Saturated.

TABLE II.
Trichomonacidal Compounds.

Chemical name	Synonym or trade name	Dilution range* killing in 10 min. but not in 5.† 1 part in:	pH	
			Test mixtures	Control
Acetylamino-hydroxyphenyl-arsonic acid	Stovarsol, Acetarsone, Spirocid	20	8.99	6.0
	Aerosol	500	5.27	6.36
Sodium methylene-sulfonamino-hydroxy-phenyl-arsonate	Aldarsone	140-150	6.2-6.3	5.9-6.1
3,3′-diamino-4,4′-dihydroxy-arseno-benzene dihydro-chloride	Arsphenamine, Salvarsan, etc.	3100-3400	5.3-5.8	6.05
Boric acid		28 (in 90 min.)	5.62	6.4
Colloidal chlorthymol	Clymocol	2200	6.28	6.36
"Meta-dihydroxy-di-secondary hexyl benzene"	Dihexylin Aqueous Tincture	5800	3.64	6.30
		5500-6000	5.0	6.57
Diiodoquinone + boric acid + lactose + dextrose	Floraquin	70% saturated (in 30 min.)	5.4-5.8	5.9
Diiodoquinone + boric acid + lactose without dextrose		70% saturated (in 30 min.)	5.6	5.9
Emetine hydrochloride		36	5.7	6.36
Isoamyl-hydrocupreine hydrochloride	Eucupin Base (in N/1 HCl adjusted)	250	5.3	6.36
1-n-hexyl-2,4-dihydroxy-benzene	Hexylresorcinol	Slight excess in emulsion	6.01	6.09
Mercuric oxycyanide		7200-7700	6.1-6.2	6.05
"Polymerized disulfonic-dioxy-dimethyl-diphenyl-methane acids"	Negatan	120-140 stock sol, or 270 active ingredients	1.76-2.16	
N′-benzoylsulfanil-amide		10% saturated (in N/1 NaOH)	10.2-10.8§	5.9
N′-furfurylsulfanil-amide		8% saturated (in N/1 NaOH)	10.7§	5.9
Sodium 3,3′-diamino-4,4′ dihydroxy-arseno-benzene-N-methanal sulfoxylate	Neoarsphenamine	2000	6.12	6.09
Ethyl hydrocupreine hydrochloride	Optochin Base (in N/1 HCl, adjusted)	142 (in 45 min.)	4.82	6.3
"P-t-octyl-phenyl-diethoxy-dimethyl-benzyl-ammonium-chloride"	Phemerol	1600	5.98	6.0
Phenol	Carbolic Acid	210	5.9-6.2	6.4
Phenylmercuric acetate		40,000	5.81	5.82
Phenylmercuric benzoate		36,000-38,000	5.8-6.0	5.84
Phenylmercuric chloride		34,000-38,000	5.8-6.0	5.99
Phenylmercuric nitrate		36,000-38,000	5.77-5.8	5.8
Quinine hydrochloride		100	6.3	6.57
Saponin		200	6.27	6.57
Silver picrate	Picrotol	14,000-17,000	6.08-6.13	6.1
Sulfadiazine Pickrell Solution	3% sulfadiazine in 8% triethanolamine	Triethanol-amine 5875		
		Sulfa-diazine 1566	8.66	5.60

TABLE II—Continued.
Trichomonacidal Compounds.

Chemical name	Synonym or trade name	Dilution range* killing in 10 min. but not in 5.† 1 part in:	pH	
			Test mixtures	Control
Sulfanilylacetamidine		400 (in N/1 NaOH)§	10.05	5.9
Sulfanilamido-thymol		250 (in N/1 NaOH)§	10.29	5.9
Disodium 3,3′-diamino-4,4′-dihydroxy-arseno-benzene-N-dimethylene sulfonate	Sulpharsphenamine‡	3000-4000	6.0-6.2	6.2
Potassium antimonyl tartrate	Tartar emetic‡	1600-2000	5.63-6.0	6.2
Sodium bismuth thioglycollate	Thiobismol‡	200-300	6.9-7.32	6.3
Trihydroxytriethyl-amine	Triethanolamine	20	9.78	
Sodium N-phenyl-glycine-amide-p-arsonate	Tryparsamide	175	6.3	6.57
5-chloro-7-iodo-8-hydroxy-quinoline	Vioform	70% saturated (30 min.)	6.3-6.5	6.57
		10% emulsion	5.88	6.57
Isoctylhydrocuprein dihydrochloride	Vuzin dihydro-chloride	2000	5.81	6.57
Zinc acetate (Analytical)		140 (45 min.)	5.22	6.3

* Slight differences in cultures necessitate expression of a killing range instead of a killing point.
† Dissolved in water unless otherwise designated in column 3.
‡ Compounds of arsenic, antimony, and bismuth gave variable results.
§ Activity may be due to the elevated pH.

bacteria-free culture[1,2] containing 2 million organisms per cubic centimeter. The population was determined by hemocytometer count and was reduced, if necessary, by dilution with sterile, modified Ringer's solution or culture fluid. Thorough mixing was then effected by gentle shaking and air agitation by means of a pipette controlled by mouth. The same procedure assured that all test organisms were washed from the upper level of the test tube wall.

At intervals of 5 and 10 minutes 0.2 cc of the test mixture containing trichomonads was transferred by a sterile pipette to 10 cc of C.P.L.M.* medium adjusted to pH 6.0.[3] (See Appendix A). Glass electrode determinations of the pH of the medication mixtures and an inoculated control containing 2.8 cc of distilled water and 1 cc of serum were then recorded.

The subcultures were examined for evidence of multiplication after 6 and 9 days. The effective concentration of the test compound is arbitrarily accepted as that dilution, or dilution range, which kills the protozoa in 10 but not in 5 minutes. Failure of the trichomonads to multiply after transfer to the C.P.L.M. medium indicated that they were killed by the test mixture.

A high protein content and an acid reaction were employed in the test mixture in order to simulate the conditions to be encountered in clinical application of the drug to the infected vagina. The validity of the test procedure is based in part upon data showing that C.P.L.M. medium* will support a high population of trichomonads after 9 days incubation when inoculated with 1 or 2 organisms. When 0.2 cc of medication mixture was transferred to the same medium the inoculum contained 2,000 treated organisms. Growth of the protozoa in the medium, as determined by inspection after 9 days incubation, indicated that more than 0.01% of the organisms had survived exposure to the medicament.

In summary the test procedure involved the addition of 400,000 trichomonads to a battery

1 Trussell, R. E., *J. Iowa M. Soc.*, 1940, **31**, 66.

2 Trussell, R. E., and Plass, E. D., *Am. J. Obst. and Gynec.*, 1940, **45**, 220.

* Cysteine-peptone-liver infusion-maltose.

3 Johnson, G., *J. Parasitol.*, 1942, **28**, 369.

of drug dilutions having in each case a volume of 4 cc and containing 25% human serum adjusted to pH 6. At intervals of 5 and 10 minutes 2,000 treated organisms contained in 0.2 cc of the test mixture were transferred to 10 cc of sterile C.P.L.M.* medium. Observation of these cultures by microscope after 6 and 9 days revealed whether or not the treated organisms had multiplied. Failure to multiply indicated that the trichomonads had been killed by exposure for a measured time interval to a given dilution of a drug.

Results. The data obtained from duplicate or triplicate tests of the same dilution range are summarized in Tables I and II.

Summary. 1. A standardized test procedure employing a highly favorable culture medium has been employed to test the trichomonacidal action of a number of recommended and suggested drugs. 2. Inspection of the data in Tables I and II suggests an approach which may lead to a more satisfactory therapeutic agent than is now available for the treatment of *Trichomonas vaginalis* vaginitis. 3. The following compounds in dilutions of 1:1000 or more killed trichomonads *in vitro* in less than 10 minutes: arsphenamine, clymocol, dihexylin, mercuric oxycyanide, neoarsphenamine, phemerol, phenylmercuric acetate, phenylmercuric benzoate, phenylmercuric chloride, phenylmercuric nitrate, silver picrate, sulfadiazine, sulfarsphenamine, tartar emetic, vuzin dihydrochloride.

APPENDIX A.

The C.P.L.M medium was prepared as follows:

32 g Bacto peptone
1.6 g Bacto agar
2.4 g cysteine monohydrochloride
1.6 g maltose[4]
320 cc Bacto liver infusion (prepared by manufacturer's instructions)
960 cc modified Ringer's solution (NaCl, 0.6%; $NaHCO_3$, $CaCl_2$, KCl, 0.01%)
Add N/1 NaOH to pH 6 (approximately 11 cc)
Keep in boiling water bath until agar is dissolved, then filter through coarse paper, add 0.7 cc 0.5% methylene blue
Tube in 8 cc volumes, autoclave, allow to cool.
Add 2 cc sterile, undiluted human serum.

The final mixture has the following percentage composition:

By volume—serum 20%, Ringer's solution 60%, liver infusion 20%.
By weight—cysteine monohydrochloride 0.15%, agar 0.10%, peptone 2.0%, maltose 0.10%, methylene blue 0.0002%.

This medium is made anaerobic[3] by the cysteine with the methylene blue serving as an indicator.

APPENDIX B.

The volume of water (H) required to dilute 2.8 cc of stock solution is determined as follows:

$$H = \frac{1.96D}{d} - 2.8,$$

where D is the final dilution desired after adding 1 cc of serum and 0.2 cc of inoculum; d is the dilution in the stock solution. After mixing, this same volume of fluid was withdrawn and discarded before adding the serum.

[4] Trussell, R. E., and Johnson, G., PROC. SOC. EXP. BIOL. AND MED., 1941, **47**, 176.

14389

Biochemical Findings in Normal and Trauma-Resistant Rats Following Trauma.

A. H. NEUFELD, C. GWENDOLINE TOBY, AND R. L. NOBLE. (Introduced by J. B. Collip.)

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A method for traumatizing rats in a rotating drum has previously been described, whereby trauma can be administered quantitatively, the percentage mortality being directly related to the degree of trauma.[1] Animals which succumb following this treatment show a picture typical of shock. By gradually increasing the amount of trauma, it was found that animals would tolerate 1000 turns, an amount fatal to the normal animal.[2] In view of the striking differences between the normal and the resistant rat subjected to trauma, chemical analyses were done on the blood and tissues of these animals.

Methods. Fed male rats weighing 200 to

[1] Noble, R. L., and Collip, J. B., *Quart. J. Exp. Physiol.*, 1942, **31**, 187.

[2] Noble, R. L., *Am. J. Physiol.*, 1943, **138**, 346.

300 g were used in all cases except when heart glycogen was determined. Here females of 140 g were used. The animals were subjected to degrees of trauma varying from 300 to 700 turns of the drum—causing from 5% to 60% mortality in animals of this size. The resistant rats had received 1000 turns at weekly intervals over a period of several weeks. In most of the experiments, following trauma the animals were anaesthetized with nembutal, the hind leg muscles were frozen *in situ* in CO_2-ether mixture by the usual procedure, and the blood was taken by heart puncture. The liver samples were removed immediately after the bleeding. In experiments in which only sugar and lactic acid determinations were done, blood samples were taken repeatedly from the tails of unanesthetized animals.

The following determinations were made. Glycogen was done by the Good, Kramer, and Somogyi method,[3] and reducing and blood sugars by Somogyi's modification.[4] Lactic acid was done by a modified Barker and Summerson procedure,[5] the muscle first being thoroughly extracted with trichloracetic acid. Pyruvic acid was determined by Klein's procedure,[6] taking into account the recommendations of Elgart and Nelson.[7] Na and K were estimated by recent modifications of the zinc uranium acetate and the silver cobaltnitrite methods.[8] Amino acids were determined by Frame, Russell, and Wilhelm's modification of the Folin Method,[9] allowance being made for the uric acid present. NPN was done on tungstic acid filtrate by the micro-Kjeldahl distillation method, and the ammonia titrated with N/100 HCl.[10] Urea nitrogen was done according to the Folin and Wu method, using micro-Kjeldahl distillation and titration as above.[11] Uric acid was done colorimetrically by the method of Benedict and Behre[12] and creatinine and creatine by the method of Folin and Wu.[13] Phosphates were determined according to Fiske and Subbarow,[14] and serum calcium according to Clark and Collip's modification of the Tisdall method.[15]

The results shown in Tables I and II were analyzed statistically, expressed as the mean and standard error, and the significance of the changes determined. (Complete data available on request.) In the normal animal following trauma a significant increase was noted in blood sugar and pyruvic acid, and in blood and muscle lactic acid, the highest values being obtained in from 15 to 30 minutes after removal from the drum. The resistant animal after trauma showed an increase in all these constituents with the exception of pyruvic acid, but the differences in response between the normal and resistant groups were highly significant. The muscle and liver glycogen values reflected the changes seen in the blood sugar and lactic acid. At 6 hours, when the blood sugar was still slightly above normal (133 mg), the average liver glycogen was 0.91% in 6 animals, while the muscle glycogen tended to return to normal (365 mg %). Heart glycogens done on 4 animals showed no change following moderate or severe trauma. A small group of normal rats showed nearly 100% increase in blood phosphate, while in the resistant animals phosphate remained unchanged or tended to fall. A similar change occurred in serum phosphates. Ca remained practically unchanged. Serum Na showed no significant change in the normal or in the resistant rat, while K showed a slight rise in the normal at 15 minutes but remained unchanged throughout in the resistant animal. Haemolysis occurred in some animals following the trauma. This may be responsible for the rise in K in the normal rat. Clarke and Cleghorn, using the same method, report

[3] Good, C. A., Kramer, H., and Somogyi, M., *J. Biol. Chem.*, 1933, **100**, 485.

[4] Somogyi, M., *J. Biol. Chem.*, 1926, **70**, 599.

[5] Barker, S. B., and Summerson, W. H., *J. Biol. Chem.*, 1941, **138**, 535.

[6] Klein, D., *J. Biol. Chem.*, 1941, **137**, 311.

[7] Elgart, S., and Nelson, N., *J. Biol. Chem.*, 1941, **138**, 443.

[8] Neufeld, A. H., *J. Biol. Chem.*, in press.

[9] Frame, E. G., Russell, J. A., and Wilhelmi, A. E., *J. Biol. Chem.*, 1943, **149**, 255.

[10] Peters, J. P., and van Slyke, D. D., 1932, *Quantitative Clinical Chemistry*, Baltimore, Vol. II. pp. 63 and 530.

[11] *Ibid.*, p. 557.

[12] *Ibid.*, p. 593.

[13] *Ibid.*, p. 604.

[14] Fiske, C. H., and Subbarow, Y., *J. Biol. Chem.*, 1925, **55**, 375.

[15] Clark, E. P., and Collip, J. B., *J. Biol. Chem.*, 1925, **63**, 461.

TABLE I.
Blood Analyses.

Blood (B) and Serum (S) Analyses, mg %		No. of turns in drum	Control	Minutes after removal from drum: 15	30	60-120
B. Sugar	N*	300	124 (11)†	140 (11)†	133 (10)†	117 (15)†
B. "	TR*	300	113 (6)	132 (6)	117 (5)	112 (6)
B. "	N	500-600	125 (22)	192 (17)	151 (9)	153 (14)
B. "	TR	500-600	128 (14)	141 (18)	141 (16)	138 (12)
B. Lactic Acid	N	300	36 (11)	135 (11)	56 (11)	42 (15)
B. " "	TR	300	32 (6)	67 (4)	42 (6)	36 (6)
B. " "	N	500-600	36 (17)	190 (21)	110 (14)	46 (8)
B. " "	TR	500-600	28 (8)	70 (10)	48 (10)	26 (8)
B. Pyruvic Acid	N	500	0.80 (6)	0.92 (6)	1.28 (6)	0.80 (6)
B. " "	TR	500	0.84 (6)	0.74 (4)	0.80 (6)	0.75 (4)
S. Sodium	N	500-600	211 (10)	208 (12)	218 (9)	209 (11)
S. "	TR	500-600	216 (6)	234 (6)	243 (6)	
S. Potassium	N	500-600	22 (10)	27 (8)	23 (11)	24 (10)
S. "	TR	500-600	20 (6)	19.8 (6)	19.8 (6)	
B. N.P.N.	N	500-700	38 (20)	56 (12)	60 (25)	63 (13)
B. "	TR	500-700	38 (8)	37 (6)	33 (8)	38 (4)
B. Urea N	N	500	14.7 (10)		21.6 (11)	
B. Uric Acid-N	N	500	0.82 (6)		1.48 (8)	
B. Creatinine-N	N	500	0.29 (10)		0.37 (11)	
B. Creatine-N	N	500	1.86 (10)		2.49 (11)	
B. Amino Acid N	N	500	9.4 (6)		13.4 (8)	
Rest N	N	500	5.4 (6)		10.2 (8)	
B. Phosphate	N	500	4.83 (3)	9.86 (3)	7.05 (3)	5.56 (3)
B. "	TR	500	4.35 (2)	4.40 (4)	3.70 (2)	3.80 (4)
S. "	N	500	5.95 (4)	10.92 (4)	9.97 (4)	
S. "	TR	500	5.92 (4)	6.15 (4)	5.57 (4)	
S. Calcium	N	500	10.2 (4)	10.77 (4)	10.87 (4)	

* N—Normal rats.
* TR—Rats made resistant to trauma as described in the text.
† No. of animals used.

TABLE II.
Tissue Analyses. 500-600 Turns of Drum.

Liver (L) and Muscle (M) Analyses, mg %		Control	Minutes after removal from drum: 15	30	60-120
L. Glycogen	N*	3520 (20)†	2610 (24)†	2280 (12)†	2220 (17)†
L. "	TR*	2410 (12)	3440 (9)	2690 (12)	1170 (3)
M. "	N	497 (14)	347 (9)	309 (14)	329 (10)
M. "	TR	529 (8)	443 (5)	477 (9)	
M. Lactic Acid	N	30 (6)	158 (10)	109 (6)	95 (4)
M. " "	TR	22 (6)	52 (4)	46 (6)	
M. Sodium	N	62 (13)	53 (11)	55 (5)	56 (11)
M. "	TR	57 (10)	43 (6)	54 (10)	
M. Potassium	N	353 (14)	364 (16)	377 (9)	340 (11)
M. "	TR	358 (10)	360 (6)	353 (10)	

* N—Normal rats.
* TR—Rats made resistant to trauma as described in the text.
† No. of animals used.

similar results.[16] There were no significant changes in either Na or K of muscle, although the Na showed a non-significant fall, and the K a rise in both groups. Non-protein N rose significantly in the normal at 15 minutes and remained elevated for at least 2 hours. As can be seen from the N partition in Table I, the increase in all the components was significant except in the case of creatinine. There was no increase in NPN in the resistant rat.

Amounts of blood withdrawn were found in control experiments not to be sufficient to

[16] Clarke, A. P. W., and Cleghorn, R. A., *Endocrinology*, 1942, **31**, 597.

produce any significant biochemical changes *per se*. Similarly, alterations in blood volume as determined by hemoglobin estimations were not related to these changes. Control experiments with adrenaline (0.25 mg per kg) produced the same elevation of the blood sugar in the resistant as in the normal animal.

The differences in blood and tissue chemistry shown by the normal and the resistant rat in response to trauma seem to indicate that the resistance which develops as a result of repeated traumatization, involved a stabilization of metabolic processes which are markedly upset in the non-resistant traumatized animal.

Summary. Biochemical changes in the blood and tissues of fed normal rats, and of rats made resistant to trauma, have been studied following the subjection of these animals to trauma in a revolving drum.

NPN rose significantly in the normal, but remained unchanged in the resistant rat.

Blood sugar, and blood and muscle lactic acid, rose in both the normal and resistant, but the difference between the two groups was highly significant. Pyruvic acid rose significantly in the normal, but showed no change in the resistant animal.

While whole blood and serum PO_4 rose 100% in the normal, Ca remained unchanged. There was no change in PO_4 in the resistant rat.

Values for Na and K of blood and muscle, and for liver, heart, and skeletal muscle glycogen showed non-significant differences between normal and resistant animals.

14390

Drug Prophylaxis against Lethal Effects of Severe Anoxia: VI. Neostigmine Bromide and Diphenylhydantoin.*

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In a preliminary survey of effects of autonomic agents on mortality of mice exposed to acute anoxic anoxia, it was noted that large doses of physostigmine and certain other cholinergic drugs tend to protect vs. anoxia while large doses of neostigmine methylsulfate do not do so and instead appear to be harmful. It was thought advisable to study this unexpected result in more detail.

Neostigmine bromide was administered intraperitoneally (ip.) or orally to 240 mice in doses listed in Table I, 30-60 min. before exposure to anoxia. The higher doses were within the lethal range: 5/20 mice treated with 0.3 mg/kg ip., 1/20 treated with 0.5 mg/kg orally, and 3/20 treated with 0.3 mg/kg ip. + diphenylhydantoin ip. died before exposure to anoxia.

Diphenylhydantoin Na was also administered to 140 mice, 110 of which had received neostigmine a few minutes before this treatment. All injections of dilantin were of 100 mg/kg ip. and were given approximately 30 minutes before exposure to anoxia. This agent has been shown by Hoff and Yahn[2] to be of value in protecting rats vs. acute anoxia and was used in the present study to test its effects in mice and also to determine whether or not a prophylactic synergy results from combination with neostigmine therapy.

Anoxia was induced by a standard method previously described.[3] Treated mice together with an equal number of control mice were exposed with adequate ventilation in a decompression chamber for 10 minutes to a reduced atmospheric pressure corresponding to an ap-

* This study was supported in part by a grant-in-aid from Hoffmann-LaRoche, Inc., Nutley, N.J.

1 Emerson, G. A., and Van Liere, E. J., *J. Lab. and Clin. Med.*, 1943, **28**, 700.

2 Hoff, E. C., and Yahn, C., *Federation Proc.*, 1943, **2**, 22.

3 Emerson, G. A., and Van Liere, E. J., *J. Lab. and Clin. Med.*, 1943, **28**, 689.

TABLE I.
Influence of Neostigmine Bromide and Na Diphenylhydantoinate on Lethal Effects of Acute Anoxia.

Neostigmine bromide		Dilantin Na		Mortality ratios		
Route	mg/kg	Route	mg/kg	Treated mice	Control mice	p
Intraper.	0.1	—	—	12/30	13/30	—
"	0.3	—	—	9/15	10/20	—
Oral	0.1	—	—	4/20	9/20	>0.05
"	0.2	—	—	2/20	9/20	<0.05
"	0.3	—	—	11/20	10/20	—
"	0.5	—	—	4/19	2/20	—
Intraper.	0.1	intraper.	100	2/30	13/30	<0.005
"	0.3	"	100	4/17	10/20	>0.05
Oral	0.2	"	100	0/40	27/40	<0.005
"	0.3	"	100	2/20	10/20	<0.05
—	—	"	100	3/30	13/30	0.005

proximate altitude of 10,000 feet, after which the pressure was further reduced at a rate corresponding to an elevation in altitude of approximately 1,000 ft/min. Exposure was usually terminated at a reduced pressure under which about half of the control mice died.

Results are noted in Table 1. Mortality ratios signify the number of mice dying during anoxia / the total number of mice exposed. Statistical treatment of the results by the exact factorial method[4] yields the values noted for p in Table 1, or the hazard that the results are due solely to chance. It may be seen that the most significant prophylaxis was obtained with combined treatment; and particularly that all of 40 mice given 0.2 mg/kg of neostigmine bromide by mouth + dilantin ip. survived anoxic conditions killing 27/40 controls exposed simultaneously.

Previous results[1] with neostigmine methylsulfate showed a slight, statistically insignificant prophylactic effect of ip. injection of 0.1 mg/kg and a definitely harmful effect of ip. injection of 0.5 mg/kg. The present results indicate a more marked prophylactic effect of oral doses of neostigmine bromide below the lethal range, with probable statistical significance at a dose of 0.2 mg/kg. With larger oral doses, however, this protective effect disappears.

Diphenylhydantoin has a definite prophylactic effect vs. anoxia in mice as well as rats, and is more effective in mice than ethanol, amytal or pentobarbital.[5] The terminal stage of anoxia in mice is usually convulsive and convulsant agents in the main do not reduce mortality.[6] It is probable that diphenylhydantoin exerts its preventive action in mice through its anticonvulsant properties, so that application of the present findings to man is not justified. Neostigmine, in large dose, acts as a convulsant; this may explain the lack of prophylactic effects above a certain optimum dose. Further, excessive salivation occurs in mice given large doses of neostigmine, especially by the ip. route; this also may play a part in increasing mortality under the physiologic handicaps of severe anoxia.

As an incidental finding, diphenylhydantoin appears to antagonize in part the lethal toxicity of neostigmine even when administered after considerable absorption of neostigmine has occurred. All deaths occurring before exposure to anoxia in mice treated with both agents were accompanied by convulsions and could be attributed to toxicity of neostigmine.

Summary. Prophylactic effects of diphenylhydantoin vs. anoxia in rats are confirmed

[4] Loewenthal, L. J. A., and Wilson, W. A., *Brit. Med. J.*, 1939, **2**, 110.

[5] Emerson, G. A., Van Liere, E. J., and Morrison, J. L., PROC. SOC. EXP. BIOL. AND MED., 1942, **49**, 376.

[6] Emerson, G. A., and Van Liere, E. J., *Arch. internat. Pharmacodyn.*, 1940, **64**, 239.

for mice also. Oral administration of 0.2 mg/kg of neostigmine bromide to mice reduces mortality in severe anoxia but larger doses are not prophylactic. Together, the 2 agents prevented death of any of 40 mice exposed to anoxic conditions simultaneously killing 27 of 40 untreated mice.

14391

Cholinergic Porphyrin Lacrymation and Paradoxical Mydriasis in the Rat. Possible Hame Nature of Choline Esterase.

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Waddell[1] first reported the pilocarpine mydriasis in the rat and attributed it to peripheral parasympathetic depression. Koppanyi and Sun[2] ascribed the paradoxical response to a ganglion paralysant action. Barnard, Tyllas and Mizock[3] could not relate the anomalous pilocarpine response to corneal anesthesia but they noticed and failed to report that acetylcholine gave the same response. Both pilocarpine and acetylcholine would provoke the secretion of "bloody tears" in a certain number of rats, particularly younger ones. Cytologic examination of the tears failed to show erythrocytes and sections of the harderian gland excised at the height of the response showed neither the expected hyperemia nor rhexis. Repeated pilocarpine administrations over a period of weeks appeared to exhaust the mechanism and this was attributed to a traumatic fibrosis but here again histologic examination of the gland failed to bear out the assumption.

The discovery of the fluorescence of this structure[4] and of the porphyrin nature of the staining material on the snouts of rats with vitamin B complex component deficiencies[5] and that the harderian gland was a porphyrin excreting organ[6] and the source of the staining material[7] redirected attention to the phenomenon of reddish tears during cholinergesis in the rat particularly since Hodge and Goldstein[8] reported as "bloody tears" the secretion observed after toxic doses of choline. A reexamination of the lacrymal secretion of rats poisoned by choline, by acetylcholine, by pilocarpine, and by cyanamide, revealed no other pigment than neutral amorphous protoporphrin.

During the course of the study with the last named drug, the nature of the paradoxical mydriasis was elicited. In doses of from 200 to 400 mg per kilo, cyanamide evokes a parasympatheticomimetic response in the rat, much more protracted in its course than that of pilocarpine, the animal surviving for several hours in coma. This protraction allows for a thorough observation of the ophthalmic findings in sustained cholinergesis. Immediately after the intraperitoneal injection of cyanamide, there is weakness and fibrillary twitching; during this stage a definite miosis is evident. In about 15 minutes, the respira-

1 Waddell, J. A., *J. Lab. and Clin. Med.*, 1926, **12**, 232.

2 Koppanyi, T., and Sun, K. H., *Am. J. Physiol.*, 1926, **78**, 358.

3 Barnard, R. D., Tyllas, H. A., and Mizock, S. L., PROC. SOC. EXP. BIOL. AND MED., 1932, **29**, 691.

4 Strong, L. C., and Figge, F. H. J., *Science*, 1941, **94**, 331.

5 Smith, S. G., PROC. SOC. EXP. BIOL. AND MED., 1942, **49**, 691.

6 Grafflin, A. L., *Am. J. Anat.*, 1942, **71**, 43.

7 McElroy, J. W., Salmon, K., Figge, F. H. J., and Cowgill, G. R., *Science*, 1941, **94**, 467.

8 Hodge, H. C., and Goldstein, M. R., PROC. SOC. EXP. BIOL. AND MED., 1942, **51**, 281.

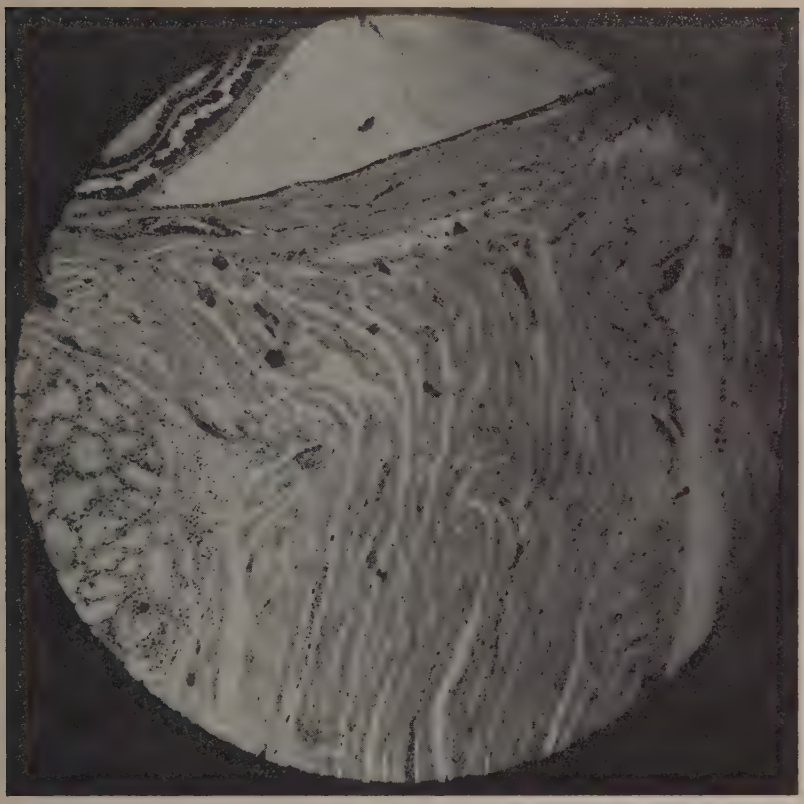

FIG. 1.

Sagittal section through optic nerve sheath of rat poisoned by cyanamide. It shows the encroachment of the engorged harderian gland on the sheath, passive hyperemia of the rim of the lamina cribrosa and disorganization of the ganglionic layers of the retina. Sections of this preparation closer to the midline show complete obliteration of the vaginal space.

tion becomes labored, frequent evacuations are noted and salivation and lacrymation become profuse. With the latter, a moderate mydriasis replaces the initial miosis. Within an hour, the animal lapses into coma with fibrillary twitching and the tearing which continues profusely becomes colorless, slightly mucoid and does not fluoresce in ultra-violet light. Death occurs in from 3 to 6 hours after injection of cyanamide. In the terminal stages of cyanamide "shock" the iris vessels are engorged and the fundus has an unusually reddish reflex. Ophthalmoscopic examination shows papilledema and retinal venous engorgement; the arteries appearing to be collapsed and relatively bloodless.

These findings comprise the ocular manifestations of raised intracranial or intraorbital pressure; there was no systemic evidence of the former but considerable indication of the latter. Heidgen and Barnard[9] in tracing the oculomotor tonus reflex pathway in the rat, found that section of the optic nerve alone would abolish the normally high pupiloconstrictor tone of this animal. Here, obviously, was the key to the cholinergic paradoxical mydriasis; increased intraorbital pressure created by the engorged harderian gland and reflected onto the optic nerve causing a conduction block in the visceral

[9] Heidgen, M. F., and Barnard, R. D., *Am. J. Physiol.*, 1931, **92**, 276.

FIG. 2.
Midsagittal section through optic nerve head of atropinized rat poisoned by cyanamide. The essential normality of all structures indicates that the changes shown in Fig. 1 are probably not a specific effect of cyanamide (carbimid) but due rather to cholinergesis.

efferent oculomotor fibres. Histologic section of the intraorbital portion of the nerve, excised with its contiguum during the later stages of cyanamide intoxication, shows, in addition to the complete investment of this portion of the nerve by the intumescent gland, the passive hyperemia distal to it. (Fig. 1).

None of the 5 animals atropinized (0.3 mg/kilo) immediately after administration of cyanamide showed any evidence of raised intraorbital pressure. Mydriasis, of course, is immediate and complete but lacrymation is absent. (The preservation of the vaginal space around the optic nerve of one of these animals is shown in Fig. 2). The coma of such an animal is as profound as that exhibited by the non-atropinized, cyanamide intoxicated ones. There is, however, no fibrillary twitching, bronchorrhea or lacrymation indicating that these are the cholinergic manifestations of the poison. One can not account for the extreme prostration of cyanamide intoxication—actually resembling in its degree that of surgical shock—on the basis of cholinergesis. While the latter seems to be the main toxicologic feature of cyanamide absorption in man, severe cases of poisoning in humans[10] also results in prostration to the shock level. A further curious propensity of human cyanamide intoxication is its aggrava-

[10] Mellinghoff, E., and Thomas, D., *Deutsch. Med. Wchnscrft.*, 1939, **65**, 1636.

tion by alcohol imbibition. The parasympatheticomimetic effects of acetylcholine are also potentiated by alcohol[11] and the pharmacologic similarity of cyanamide to the parasympathetic hormone along with certain distinctive chemical features of the former permits the postulation that choline esterase is a heme compound and even enables us to assign to it a structure similar to that of the iron-porphyrin complex in ferrihemoglobin. For cyanamide, like cyanide, combines with the iron of ferriheme in either or both of two distinct types of linkages; (A), the polar covalent or ferrideheme linkage and (B), by coordination; the hemochromogen linkage. (A) appears to be the pharmacodynamically important linkage since the heme compounds as they exist physiologically are already in the (B) form and the amino acids, while they are suitable hemochromogen formers (Bertin-Sans and deMontessier[12]) have little pharmacologic effect. Thiamine, which does coordinate with ferriheme[13] and which potentiates acetylcholine, does not act on the esterase.[14] The ferrideheme formers, on the other hand are all very active pharmacologically and both on the basis of this activity and their chemical behavior, may be separated into two categories:

1. Those which form ferridehemes with a very low degree of dissociation, *e.g.*, azide, cyanide, fluoride, fulminate and hydrosulfide. These are predominantly tissue asphyxiants through their affinity for the ferriheme component of the cytochrome system. If cholinergesis attends their advent in the organism it is masked by their more rapid and dramatic toxic effects.*

2. Those which differ quantitatively from (1) by their larger ionic diameter and the higher degree of dissociation of the ferridehemes which they form. Cyanamide, cyanate nitrite and thiocyanate make up the group and while they have some tissue asphyxiant action they are mainly depressor drugs.

On the assumption of a ferrihemoglobin type of structure for choline esterase, the depressor activity of these drugs might be explained. Ferrideheme formation with any of the members of category (2) would prevent the labile valence change of the iron by which the activity of many of the heme compounds is conditioned. That cyanamide inhibits the decomposition of cetyl pyridinium chloride by ferriheme has been shown.[13] There remains to be explained on the basis of this assumption the cholinergic effects of physostigmine and of choline; the latter not being a ferrideheme former. Likewise to be explained is the anti-esterase potentiation of acetylcholine by ethanol.[11] The case of choline may be one of enzymatic synthesis of acetylcholine. Physostigmine may be a ferrideheme former through acid dissociation of its secondary imine hydrogen; spectroscopic studies designed to demonstrate such a combination with ferrihemoglobin have been inconclusive; the pigment is partially denaturated and the alkaloid, itself, becomes colored in solution. One cannot rely on spectroscopic evidence for the negative case in this connection, because fluoride does not modify the color of cetyl pyridinium ferrihemochromogen but does, like cyanamide, inhibit its disruption. A spectroscopically indistinctive combination with ferrihemoglobin is that of ethanol; the latter having anti-esterase activity.[11] Non-ferrideheme combinations with the iron of ferrihemoglobin of iminazolyl groups and of neutral moieties (ammonia, ethanol) have been described.[16,17] Coryell and Stitt[17] report a significant change in the magnetic susceptibility from that of ferrihemoglobin in its ethanol derivative. This change would portend an alteration in the fer-

[11] Ettinger, G. H., Brown, A. B., and Megill, A. H., *J. Pharm. and Exp. Therap.*, 1941, **73**, 119.

[12] Bertin-Sans, H., and deMontessier, J., *C. R. Soc. de Biol.*, 1892, **114**, 923.

[13] Barnard, R. D., *J. Am. Pharm. Assn.*, in press.

[14] Byer, J., and Harpruder, K., *J. Pharm. and Exp. Therap.*, 1940, **70**, 328.

* Choline esterase is inactivated by fluoride ion *in vitro* (Nachmansohn[15]).

[15] Nachmansohn, D., *C. R. Soc. de Biol.*, 1939, **130**, 1065.

[16] Russell, C. D., and Pauling, L., *Proc. Nat. Acad. Science*, 1939, **25**, 517.

[17] Coryell, C. D., and Stitt, F., *J. Am. Chem. Soc.*, 1940, **62**, 2951.

riheme prosthetic nucleus and were it to occur in one of the components of a delicately poised enzyme system might modify its activity.

The only other suggestion as to the chemical nature of choline esterase has resulted from the investigations of Nachmansohn and Lederer.[18] They concluded that its activity was conditioned by a sulfhydryl group because of its inactivation by heavy metal ions; treatment with reduced glutathione was followed by reactivation. The same facts, however, can be cited in support of the ferriheme theory of its constitution. Ferrihemoglobin forms well defined compounds with each of the ions of the b family of the heavy metals of Groups I and II of the periodic system[19] (these incidentally also form insoluble sulfides) and the metalloferrihemoglobin combination is broken by its treatment with any reducing agent.

The hypothesis of the ferriheme nature of choline esterase and its corollary, that the enzyme may actually be a cytochrome, peroxidase, catalase, ferrihemoglobin and/or myomethemoglobin will have its scrutiny hampered by the universal distribution of iron compounds in living tissues.

[18] Nachmansohn, D., and Lederer, N., *Bull. Soc. Biol. Chim.*, 1939, **21**, 797.

[19] U. S. Patent No. 2,296,377 (assigned to Armour and Company).

14392

Antibacterial Filtrates from Cultures of *Aspergillus flavipes.*

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The production of a powerful, non-toxic antibacterial substance by *Penicillium notatum* has stimulated search in many quarters for other molds showing similar behavior. In addition to the work with *Aspergillus flavus* already reported,[1] the author has examined about 30 molds. Most of them were aspergilli, obtained from the collection of Dr. Charles Thom. A number have shown antibacterial activity of varying degree, but one, *Aspergillus flavipes*, has been of particular interest. As this work has been interrupted, it seems appropriate to record the results at this time.

The mold was designated in the Thom collection "175.4303.46-Texas soil." As received on an agar slant there was a heavy, tight mycelial growth which could be broken with difficulty, and spores for transfer were not easily obtained from this part of the growth. However, a light sandy area of sporulation was present at the tip of the slant where it was thinnest. This type of growth persisted in subcultures on slants, but by growing on Czapek-Dox agar in a Petri dish an even, sandy growth with ample sporulation was obtained. The growth at room temperature is colorless for about 3 days, then takes on a light brown sandy appearance as sporulation develops, and after 4 or 5 days a characteristic camphor-like odor is noticed. This odor has been developed only on solid media.

Little or no antibacterial activity was noted when the mold was grown on Czapek-Dox solution, but it appeared when a protein digest was used. With this medium (Tryptone-Difco) the best results were noted without any adjuvants at all, and sugar was definitely deleterious. The best medium of all, however, consisted solely of corn steep liquor, in the concentration of 5 or 10 ml to 100 ml of water. There was no definite advantage in the

* This work was done while the author was a guest in the Department of Bacteriology, with the aid of a grant from the Fluid Research Fund of the Rockefeller Foundation. The author gratefully acknowledges the helpful interest of Dr. J. Howard Brown, Dr. Ruth Wichelhausen, and Mrs. Lucille B. Robinson.

[1] White, E. C., and Hill, J. H., *J. Bacteriology*, 1943, **45**, 433.

higher concentration. This strongly acid solution was neutralized, and the precipitate formed on autoclaving was disregarded. Some variation was noted in the results with steep liquor of different origins, and results here recorded were obtained with corn steep liquor secured from the Corn Products Refining Co.

The 5- to 6-day filtrate, which was always alkaline, was neutralized, filtered through paper, clarified by centrifugalization if necessary and Seitz filtered. This filtrate was serially diluted with infusion broth, or, when *Strep. pyogenes* or a pneumococcus was the test organism, with broth containing 3% ascitic fluid. Further addition of one drop of defibrinated rabbit blood per ml did not alter the results, showing that presence of small amounts of blood does not interfere with the activity. One ml of each dilution (made in two-fold steps) was inoculated with a 4 mm loopful of the appropriate bacterial culture and incubated for 24 hours. In the case of *Cl. welchii* the incubation time was 48 hours in the anaerobic jar. The highest inhibitory dilutions noted are shown in the table. Although lower limits were found at times, the highest limits as recorded on the table were those generally observed. Indeed, the behavior of these filtrates has been more regular than those of any other mold worked with, including *P. notatum*. Within the period of observation, about 9 months, the constancy of appearance of the stock cultures and the constancy of antibacterial activity of the filtrates have suggested that the spontaneous formation of variants noted with other molds has been absent here.

TABLE I.

Inhibitory Dilutions of Filtrates from *A. flavipes* Grown on Diluted Corn Steep Liquor.

Organism	Inhibitory titer of filtrate
Strep. pyogenes C 203. (Old culture transferred daily for nearly a year, without mouse passage)	1280
Strep. pyogenes C 203. A virulent culture, from a recent mouse passage. (Obtained from Dr. Eleanor Bliss)	320
Pneumococcus, Type III, recently isolated from a case of meningitis	640
Pneumococcus, Type IV	1280
Cl. welchii, 24-hr growth in thioglycollate broth	640
Cl. welchii spores, 14-day growth anaerobically in sugar-free broth	640
Staph. aureus	5 to 10
Strep. fecalis	0 to 5
E. coli	No activity
A. aerogenes	" "

Without the isolation of a highly purified product one can only speculate about the nature of the antibiotic substance formed by *A. flavipes*. However, the pattern of behavior of these filtrates resembles that of filtrates from *P. notatum* tested during the course of this work, using the same organisms with both filtrates. The similarity is particularly noted in the case of *Staph. aureus*, where, with both molds, the activity was very low as compared to that against other Gram positive organisms. Filtrates from *A. flavipes* tested against *Staphylococcus aureus* by the Oxford cup and plate method showed zones of inhibition as wide as 25 mm in diameter. When the filtrate was diluted with one, 2 or 3 parts of broth, the zones were not narrowed proportionately, but only reduced to 22, 18 and 15 mm respectively. With this strain of staphylococcus the zones of inhibition were generally narrower with crude penicillin filtrates than with *A. flavipes* filtrates.

Preliminary experiments showed that the active material was largely, but not completely removed by charcoal at pH 4.0. No success was had in attempts to elute the material with buffers and various solvents. As much as 50% of the activity was recovered by concentrating the filtrates *in vacuo*, precipitating with 10 volumes of acetone and evaporating the solvent after filtering off the gummy precipitate.

The isolation of penicillin-like products from the author's strain of *A. flavus*[2] and from another strain of *A. flavus* by Bush and Goth,[3] together with the results here reported, suggest that further search may reveal that substances identical with penicillin or closely related products may be formed fairly commonly by aspergilli.

[2] McKee, C. M., and MacPhillamy, H. B., PROC. SOC. EXP. BIOL. AND MED., 1943, **53**, 247.

[3] Bush, M. T., and Goth, A., *J. Pharm. and Exp. Therap.*, 1943, **78**, 164.

14393

A Comparison of Survival to Decompression in Air and in Oxygen.

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It is generally assumed that death due to ascension to high altitudes or simulated high altitudes by decompression is due to anoxia entirely. Since human alveolar air is approximately saturated with water vapor the pH_2O at body temperature (37°) is about 47 mm Hg. The alveolar pCO_2 is ordinarily assumed to be about 40 mm Hg. Therefore the minimal pressure of 87 mm would be required before any oxygen would be available for respiration.[1,2]

The amount of water and carbon dioxide dilution of alveolar air would be influenced by the rate of ventilation of the lungs and in small animals such as rats and mice hyperventilation would have a significant effect on pCO_2 and pH_2O. Hailman[3] has shown that hyperventilation raises the altitude at which respiration fails in rats. He suggests that this is due to the reduction of pCO_2 and pH_2O by hyperventilation and from a possible fall in body temperature as a result of anoxia.

Many factors are known to influence survival to anoxic anoxia besides the pO_2, including the age of the animal,[4] rate of ascent (or decompression).[5,6] Armstrong and Heim[5] reported that higher altitudes can be reached before death if a rapid ascent is made. In our experiments (unpublished) we have found a greater tolerance to hypoxia in mice decompressed slowly as compared to those decompressed rapidly unless the decompression is extremely rapid ("explosive decompression".)

In this investigation white mice of approximately the same age and of about 17 g average body weight (males and females) and baby chicks of the same age (all males) were used in a decompression chamber (bell jar) which communicated with a large ballast jar which in turn was connected to a vacuum pump. The pressure inside the animal chamber was carefully controlled by a Hg manometer. The rate of decompression was regulated by an adjustable inlet valve admitting air or oxygen to the animal chamber. After various trials with different rates of decompression both continuous and discontinuous it was decided to standardize the technic to a rate of decompression shown in Table I, A from a starting pressure of 740 mm down to the critical pres-

TABLE I.

A. Rate of decompression in oxygen and in air:

Time in min:	0	3	6	9	12
Barom. press. mm Hg:	740	339	172	98	58

B. Barometric pressures at which animals died (mm Hg.):

Mice		Chicks	
In air	In oxygen	In air	In oxygen
166	83	212	90
160	76	258	90
154	94	220	88
150	87	218	89
170	70	212	87
167	69	190	93
147	67		
136	65		
142	95		
139	94		
161	75		
147	70		
176	74		
170	71		
147	76		
146	80		
171	76		
166	79		
185	74		
172	76		
Avg 158.6	70.7	218.3	89.5

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1 Armstrong, H. G., *Principles and Practice of Aviation Medicine*, 1939, Baltimore, Williams and Wilkins.

2 Horvath, S. M., Dill, D. B., and Corwin, W., *Am. J. Physiol.*, 1943, **138**, 659.

3 Hailman, H. F., Proc. Soc. Exp. Biol. and Med., 1943, **53**, 221.

4 Fazekas, J. F., Alexander, F. A. D., and Himwich, H. E., *Am. J. Physiol.*, 1941, **134**, 381.

5 Armstrong, H. G., and Heim, J. W., *J. Aviat. Med.*, 1938, **9**, 45.

6 Emerson, G. A., and Van Liere, E. J., *J. Lab. and Clin. Med.*, 1943, **28**, 689.

sure at which the animal either died or collapsed. Using chicks, the pressure at which the animals fell backward was decided upon as the critical pressure since this pressure is very close to death and represents a definite end-point. The pressure at which the last extensor thrust of the hind legs occurred was chosen as the end-point in death of the mice.

Using these two criteria as end-points chicks singly and mice in pairs were decompressed in atmospheres (1) of air and (2) of oxygen. To make certain that the animal jar contained as nearly pure oxygen as possible for the latter experiment it was washed out with approximately 14 times its own volume with oxygen and then a constant stream of oxygen passed through it during the trial. Samples of gas in the chamber were also analyzed with a Haldane apparatus to make certain of oxygen saturation. Since the rate of decompression was kept the same in all experiments by an inlet valve it can be seen that the same rate of inflow of air or oxygen existed throughout all the trials. Therefore at any given barometric pressure the only difference in the animal jar was the pO_2. All experiments were conducted at room temperature of 26°C. Each experiment was started at 740 mm Hg pressure and time was allowed for the animals to become adapted to the starting pressure. It is doubtful if this precaution was necessary because the natural barometric pressure varied between 741 and 751 and therefore there was no significant pO_2 difference.

An examination of the data (Table I, B) shows a considerable species difference in the anoxic resistance of mice and chicks. Wright[7] computes that 110 mm is the lowest barometric pressure at which a person breathing pure oxygen can remain conscious. He calculates that hyperventilation by washing out CO_2 from the blood could reduce alveolar pCO_2 from 40 to 23 mm. It has been stated[8] that pO_2 of alveolar air at the time of death is probably about 10 mm Hg. The very rapid polypneic breathing of mice undoubtedly accounts partly for their ability to tolerate lower barometric pressures than the chicks. Hailman's[3] data for rats decompressed in oxygen compare favorably with ours for mice, there being only about 5 mm difference at death.

Summary. Mice and chicks were decompressed in atmospheres of (1) air and (2) oxygen and the critical pressures determined below which the animals could not survive. A considerable species difference exists.

7 Wright, S., *Applied Physiology*, 1941, N. Y., Oxford Univ. Press.

8 Selladurai, S., and Wright, S., *Quart. J. Exp. Physiol.*, 1932, **22**, 233.

14394 P

Relation of Number of Injections to the Titer of Sperm Iso-agglutinins in Mice.*

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It is well known that certain animals can form antibodies against their own spermatozoa[1] or against the spermatozoa of other individuals of the same species.[2] In the case of the species heretofore used (guinea pigs, rabbits, rats) relatively few injections have suf-

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1 Metalnikoff, S., *Ann. Inst. Pasteur*, 1900, **14**, 577.

2 Henle, W., *J. Immunol.*, 1938, **34**, 325.

TABLE I.
Titers of Sperm Agglutinins Produced by the Injection of Mouse Sperm Into Mice.

Total No. of inj.	Approx. No. of sperm per inject., × 1,000,000	Injections	No. of ♀ mice inj.	Titers of sperm antisera*
(a) Intraper.				
4	30	every other day	1	<1:3
4	90		1	1:3
4	270		3	1:3 ♀ died ♀ died
5	30		1	<1:3
5	90		3	<1:3 <1:3 <1:12
7	30		1	1:3
7	90		1	1:48
12	3 of 10 3 of 20 3 of 30 3 of 50	Wed., Thurs., Fri.	5	<1:24 <1:24 1:6 1:24 1:48
15	30		10	1:48 1:48 1:48 1:48 1:96 1:192 1:192 1:384 1:384 >1:1536
21	10		5	1:48 1:192 1:192 1:384 1:384
21†	18 of 10 3 of 30		4	1:384 1:384 1:768 1:768
20-21	30		7	1:96 1:768 1:768 1:768 1:1536 1:1536 1:3072
21	90		1	1:48
(b) Subcut.				
15	35	Wed., Thurs., Fri.	5	<1:3 <1:3 <1:3 <1:3 <1:3

* Defined as the highest dilution of antiserum giving ++++ agglutination.
† First 4 injections were intravenous.

ficed for the formation of antibodies. The experiments with mice here reported show that for this species repeated injections are necessary for the formation of anything other than very low titers, and that with the use of repeated injections high titers of sperm iso-agglutinins can be developed.

Methods. Sperm for injection were obtained by killing male mice, dissecting out the vasa deferentia and epididymedes, mincing these in Locke's in a watch glass with fine scissors, straining through 2 fine wire strainers (75 and 112 wires to the inch respectively) into a filter flask, centrifuging to remove excess fluid and resuspending in the amount of Locke's desired for injection. The sperm yield as determined by counts with a hemocytometer ranged from about 18,000,000 to 50,000,000 per male, the average being about 30,000,000. Most injections were intraperitoneal, a few subcutaneous or intravenous. Injections were made every other day, or in courses of 3 consecutive days followed by 4 days' rest.

The males used to provide sperm were predominantly from the C57 black or BBC strains, the latter being a hybrid derivative of the C57 black strain, but some animals of other stocks were used. The animals injected were in every case B alb C (MacDowell-Bagg albino) females. They were killed 7 to 14 days after the last injection.

Titer tests were run in 1 cc vials. Sperm were obtained by stripping vasa deferentia into Locke's, 0.6 to 0.8 cc of Locke's being used for each 2 vasa. After shaking, 0.1 cc of the resulting sperm suspension was added to 0.2 cc of each serum dilution. Readings were made after 15 to 30 minutes from a drop under the microscope. The degree of agglutination was classified as 0, +, ++, +++, or ++++, the latter indicating that all or nearly all of the active sperm were stuck, usually by the tail or middle-piece, to other sperm. The activity of the sperm was invariably unimpaired as compared with the controls so that the usual picture consisted of clusters or mats of actively beating but firmly enmeshed sperm.

Results. The results show that when 7 or fewer injections were given the sera were usually negative, though a few titers of 1:3 and one of 1:48 were obtained. Twelve injections over a period of 4 weeks gave titers of 1:48 or lower. However, 15 injections over

a period of 5 weeks gave titers of 1:48 to 1:384 (and one exceptional titer of >1:1536) and 21 injections over a period of 7 weeks gave titers of 1:48 to 1:3072, the majority being 1:192 to 1:768.

The optimum dose appears to be from 10,000,000 to 30,000,000 sperm per injection. Two of 3 animals injected with 270,000,000 sperm per injection died after the fourth injection. One animal given 21 injections of 90,000,000 sperm each over 7 weeks yielded serum with the low titer of 1:48.

Subcutaneous injections failed to produce agglutinins.

All controls were negative except that one control serum was found to have a titer of 1:96. Also one lot of pooled serum from 18 untreated males used in another experiment contained sperm agglutinins. The reason for these rare agglutinins in sera of untreated animals is unknown.

Absorption tests with some of the sera produced in the above series of experiments have yielded evidence of the existence of an antigen or antigens in the sperm of C57 black mice not present in the sperm of B alb C mice. These tests will be described in detail elsewhere.

Summary. Four or 5 intraperitoneal injections on alternate days of mouse sperm into mice failed to produce more than a very low titer of sperm agglutinins. However, with 21 injections over a period of 7 weeks titers of 1:48 to 1:3072 were developed. Subcutaneous injections failed to lead to the formation of antibodies.

14395

A New *Salmonella* Type: *Salmonella mississippi.**

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Among a lot of *Salmonella* cultures received from the Mississippi Department of Health was one isolated from a stool specimen of a normal food handler. This bacillus was identical with none of the previously described *Salmonella* types and to it was assigned the name *Salmonella mississippi*. It was a motile rod which in its morphological, cultural, and biochemical characters conformed to the usual pattern of paratyphoid bacilli. Glucose, arabinose, xylose, rhamnose, maltose, dulcitol, and sorbitol were promptly fermented with the production of acid and gas. Lactose, sucrose, salicin, adonitol, and inositol were not attacked. The bacillus fermented d-tartrate, mucate, and citrate but did not utilize l-tartrate. Large amounts of hydrogen sulphide were produced in peptone broth but indol was not formed.

When examined serologically the organism was agglutinated strongly by O serums derived from *S. worthington* (I, XIII, XXIII) and *S. poona* (XIII, XXII). It was agglutinated to a lesser degree by serums which contained agglutinins for antigen I. When tested with single factor serums for antigens XXII and XXIII, agglutination occurred in both. Absorption of *S. worthington* O serum with *S. mississippi* resulted in a complete removal of agglutinins, but the bacillus did not remove all agglutinins from *S. poona* O serum. When an O serum was prepared from *S. mississippi* it was found that neither *S. poona* nor *S. worthington* was able to exhaust it of agglutinins, although when used in combination the organisms left only a slight residual titer in the serum. It is obvious that the O antigens of *S. mississippi* are complex and that the O antigens of the previously described members of the *S. poona-S. worthington* group cannot be fully expressed by the symbols hitherto assigned to

* The investigation reported in this paper is in connection with a project of the Kentucky Agricultural Experiment Station and is published by permission of the Director.

them. The O antigens of *S. grumpensis,* an undescribed type of Hormaeche and Peluffo,[1] while unlike those of *S. mississippi,* also occupy an intermediate position between *S. poona* and *S. worthington.* In view of the present lack of agreement as to how these antigens shall be designated, and since *S. mississippi* possesses all the O antigens of *S. worthington* as well as additional factors, its O antigens are labelled simply as I, XIII, XXIII. . .

When the H antigens of *S. mississippi* were examined the organism was found to be diphasic. Phase 1 was flocculated to the titer of serum derived from phase 1 of *S. paratyphi B* (b) and removed all H agglutinins from the serum when used as antigen in absorption tests. The antigens of phase 1 of *S. mississippi* may be denoted by the symbol "b." Phase 2 of the bacillus was flocculated by serums derived from all the non-specific phases of the Kauffmann-White classification. When tested with single factor serums for antigens 2, 3, 5, 6 and 7, agglutination occurred in 5 serum and to a lesser degree in 3 serum. The organism was used to absorb agglutinins from serum derived from phase 2 of *S. choleræ-suis* (1,5 . . .). After absorption a slight residue of agglutinins remained for phase 2 of *S. choleræ-suis* but the serum would no longer agglutinate phase 2 of *S. thompson* (1,5 . . .). The antigens of phase 2 of the bacillus may be expressed as 1,5 . . . The antigenic formula of *S. mississippi* is I,XIII,XXIII . . .:b-1,5 . . .

Summary. A new *Salmonella* type which was isolated from the stool specimen of a normal food handler was described. The organism was designated as *S. mississippi* and was assigned the antigenic formula I,XIII,XXIII . . .:b-1,5 . . .

[1] Hormaeche, E., and Peluffo, C. A., 1943, personal communication.